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ENZYMES OF FATTY ACID METABOLISM

III. BREAKDOWN AND SYNTHESIS OF β-KETO FATTY ACIDS*

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Breusch (1) and Wieland and Rosenthal (2) found independently that homogenates of kidney and other tissues formed citrate aerobically from acetoacetate and oxalacetate. The latter authors noted that 2 molecules of citrate were formed per molecule of acetoacetate disappearing. Breusch reported further (3, 4) that β-keto acids other than acetoacetate yielded citrate along with the fatty acid shorter than the original β-keto acid by 2 carbon atoms. Hunter and Leloir (5) found a specific α-ketoglutarate requirement for citrate synthesis from acetoacetate and oxalacetate in dialyzed kidney homogenates. Under these conditions, citrate synthesis occurred anaerobically as well as aerobically. The need for concomitant oxidation (or dismutation) of α-ketoglutarate appeared not to be related to the generation of energy-rich phosphate, since α-ketoglutarate could not be replaced by ATP.[1] Stern and Ochoa (6) showed that, in the presence of ATP, CoA, and oxalacetate, β-keto fatty acids (acetoacetate, β-ketovalerate, β-ketocaproate, and β-ketooctanoate) formed citrate on incubation with pigeon liver extracts. These results indicated (*a*) that cleavage

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[1] The following abbreviations are used: adenosine triphosphate, ATP; oxidized and reduced diphosphopyridine nucleotide, DPN^+ and DPNH; coenzyme A (reduced), CoA, HS-CoA, or CoA-SH; S-acyl coenzyme A derivatives, acyl-S-CoA or acyl CoA; glutathione (reduced), GSH; phosphotransacetylase, transacetylase; tris(hydroxymethyl)aminomethane, Tris.

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of the β-keto acids yields acetyl CoA and (*b*) that in pigeon liver extracts cleavage is preceded by an activation of the keto acid involving ATP and CoA.

A different type of activation was disclosed by experiments with enzyme fractions from pig heart (7). These experiments provided evidence for the occurrence of Reactions 1 to 3. Reaction 3 is catalyzed by the condensing enzyme (8). Succinyl CoA for Reaction 1 could be supplied either

(1) Succinyl-S-CoA + acetoacetate $\rightleftharpoons$ acetoacetyl-S-CoA + succinate

(2) Acetoacetyl-S-CoA + HS-CoA $\rightleftharpoons$ 2 acetyl-S-CoA

(3) 2 Acetyl-S-CoA + 2 oxalacetate + $2H_2O \rightleftharpoons$ 2 citrate + 2HS-CoA

by α-ketoglutarate oxidation or dismutation (9, 10), thereby explaining the results of Hunter and Leloir, or by the reaction between succinate, ATP, and CoA, catalyzed by the P enzyme (11, 12). In order to obtain citrate synthesis from acetoacetate and oxalacetate, pig heart preparations containing the enzymes catalyzing Reactions 1 and 2, as well as condensing and P enzymes, had to be supplemented (7) with (*a*) α-ketoglutarate, CoA, DPN^+, Mg^{++}, and α-ketoglutaric dehydrogenase, (*b*) succinate, ATP, CoA, and Mg^{++}, or (*c*), as found later, succinyl CoA. Acetoacetate could be replaced by β-ketovalerate, β-ketocaproate, and β-ketoisocaproate, in order of decreasing effectiveness, but not by β-ketooctanoate or β-ketoadipate. Reversibility of Reactions 1 and 2 was indicated by the fact that the pig heart system could synthesize acetoacetate from acetyl CoA but only in the presence of added succinate. In these experiments acetyl CoA was generated enzymatically from acetyl phosphate with catalytic amounts of CoA and transacetylase (13). Such a system requires that CoA be constantly regenerated and depends on the presence of succinyl CoA deacylase (11, 14) for maximal reaction rates. In accordance with this, acetoacetate synthesis was highest when the pig heart system was supplemented with both succinate and succinyl CoA deacylase (7). The need of Reaction 1 for acetoacetate synthesis by the pig heart system is due to the absence of an acetoacetyl CoA deacylase and to the equilibrium position of Reaction 2 favoring cleavage rather than synthesis of acetoacetyl CoA.

Similar evidence for the occurrence of Reactions 1 and 2 was obtained independently by Green *et al.* (15) who regarded the two reactions as possibly catalyzed by a single enzyme. We have briefly reported (16) the separation of two enzyme fractions from pig heart extracts catalyzing Reactions 1 and 2 and presented evidence for the intermediary formation of acetoacetyl CoA. The enzymes involved will be referred to (17) as CoA transferase (or simply transferase) and thiolase, respectively.[2] The pres-

[2] A Committee on Nomenclature, 2nd International Conference on Biochemical Problems of Lipides, Ghent, Belgium, July, 1955, has proposed the systematic names

ent paper supplements preliminary accounts (7, 16) of work which led to the separation and identification of the transferase and thiolase. The purification and properties of these enzymes, which have been reviewed (18, 19), will be the subject of succeeding papers of this series.

Enzymatic Cleavage of β-Keto Fatty Acids—The stoichiometry of the conversion of acetoacetate to citrate is illustrated in Table I. It may be seen that, in agreement with Reactions 1 to 3 and in confirmation of previous results with tissue homogenates (2, 5), 2 molecules of citrate are formed per molecule of acetoacetate disappearing. A crude pig heart enzyme fraction, the source of P enzyme, transferase, and thiolase, was supplemented with crystalline condensing enzyme. Succinyl CoA was generated from succinate, ATP, and CoA, by the P enzyme present. A control sample without succinate served to correct for the acetoacetate disappearing by

TABLE I

Stoichiometry of Enzymatic Conversion of Acetoacetate to Citrate

The complete system contained (in micromoles) Tris-HCl buffer, pH 7.5, 100; $MgCl_2$, 8; GSH, 10; ATP, 10; CoA, 0.1; succinate, 100; acetoacetate, 10.6; oxalacetate, 20; pig heart 20–40 ethanol fraction (prepared as described under "Methods") with 14 mg. of protein; and 0.015 mg. of crystalline condensing enzyme. Final volume 1.66 ml. Incubation, 60 minutes at 38°. Results given in micromoles.

	Acetoacetate removed	Citrate formed
Complete system	3.67	4.66
No succinate	1.34	0
Corrected	2.33	4.66

side reactions, mainly decarboxylation. No citrate was formed in the absence of succinate. In the absence of oxalacetate and with stoichiometric amounts of CoA, it can be shown that acetoacetate is indeed cleaved to yield acetyl CoA. In a typical experiment, a pig heart enzyme fraction (2.5 mg. of protein), similar to that used in the experiment of Table I, was incubated for 30 minutes at 38° with ATP (10 μmoles), CoA (1 μmole), succinate (50 μmoles), and acetoacetate (50 μmoles), in the presence of Tris-HCl buffer (50 μmoles), pH 7.5, $MgCl_2$ (2.5 μmoles), and GSH (5 μmoles). The protein was then precipitated by heating for 1 minute at 100° and pH 4.5. To the mixture were then added potassium phosphate buffer (100 μmoles), pH 7.4, potassium oxalacetate (20 μmoles), and crystalline condensing enzyme (0.03 mg.), and citrate was determined after further incubation for 30 minutes at 25°. 0.65 μmole of citrate was found, indicating that this amount of acetyl CoA had accumulated during the pre-

acetoacetylsuccinic thiophorase and acetoacetyl thiolase for transferase and thiolase, respectively.

ceding incubation period. Only traces of citrate were found in a control experiment without acetoacetate. Chromatography on paper after Stadtman (13) of an aliquot of the reaction mixture at the end of the first incubation period gave a thio ester spot with R_F 0.61.

Substitution of β-ketovalerate or β-ketocaproate for acetoacetate in the above system, in the absence of oxalacetate, leads to the production of acetyl CoA and propionyl CoA or acetyl CoA and butyryl CoA, respectively. These products were identified by paper chromatography of the hydroxamic acids according to Stadtman and Barker (20). The results

TABLE II

Chromatographic Identification of Acyl-S-CoA Compounds Formed by Enzymatic Cleavage of β-Keto Acids

The reaction mixture was identical to that described in the text for the experiment with acetoacetate except that acetoacetate was omitted and H_2S (20 μmoles) was substituted for GSH. 50 μmoles of β-ketovalerate or of β-ketocaproate were added as indicated. After incubation for 30 minutes at 38°, 0.5 volume of neutralized 2.0 M hydroxylamine solution was added and incubation was continued for 10 minutes. The protein was precipitated with excess ethanol and the hydroxamic acids were concentrated and identified by paper chromatography (20).

Hydroxamic acid	R_F			
	Authentic samples	After incubation		
		Without β-keto acid	With β-ketovalerate	With β-ketocaproate
Succino-	0.35	0.30		
Aceto-	0.52		0.50	0.52
Propiono-	0.65		0.66	
Butyro-	0.76			0.73

are shown in Table II. These experiments provided the first unequivocal demonstration of the enzymatic activation and cleavage of β-keto fatty acids to yield either acetyl CoA alone (acetoacetate) or acetyl CoA and the acyl CoA derivative shorter by 2 carbon atoms than the original β-keto acid.

Fractionation of Enzymes—The starting material consisted of either (*a*) an extract of pig heart acetone powder prepared by rubbing the powder at 25° in a mortar with 10 volumes of 0.1 M potassium phosphate buffer, pH 7.0, containing 0.001 M potassium L-cysteine, and clarified by filtration, or (*b*) an ammonium sulfate fraction between 50 and 80 per cent saturation, prepared from extracts of fresh pig heart obtained by the procedure of Kaufman *et al.* (11), and dialyzed against 0.02 M potassium phosphate

buffer, pH 7.0, containing 0.001 M L-cysteine. The starting solution was brought to 40 per cent saturation with solid ammonium sulfate added slowly with mechanical stirring at 0–3°. The precipitate was removed by centrifugation in the cold room at 18,000 × *g* and discarded. The supernatant solution was brought to 60 per cent saturation with ammonium sulfate, based on the original volume; the precipitate was collected by centrifugation as above, dissolved in 0.02 M potassium chloride, and dialyzed against the same solution in the presence of 0.001 M L-cysteine for 18 hours (40–60 ammonium sulfate fraction).

The 40–60 ammonium sulfate fraction was diluted with 0.02 M potassium chloride to bring the protein concentration to about 10 mg. per ml. Chilled ethanol (−30°) was then added dropwise with mechanical stirring to a concentration of 20 per cent by volume, the temperature being lowered to −4°. The precipitate was collected by centrifugation at −4°, suspended in 0.02 M potassium chloride, and dialyzed against the same solution adjusted to pH 7.0 and containing 0.001 M L-cysteine. This will be referred to as Fraction A. 0.8 M zinc acetate was added to the clear supernatant fluid at −4° to make the final concentration 0.02 M. The precipitate was collected by centrifugation at the same temperature and suspended and dialyzed as the previous one. This will be referred to as Fraction B. Any precipitate remaining in Fractions A and B after dialysis was removed by centrifugation at 0° and discarded.

Breakdown and Synthesis of Acetoacetate by Enzyme Fractions—Through the above fractionation, transferase and thiolase could be largely separated from each other. As shown in Table III, either Fraction A or B by itself was relatively incapable of bringing about the conversion of acetoacetate to citrate or that of acetyl CoA (generated with transacetylase from acetyl phosphate and catalytic amounts of CoA) to acetoacetate, while there was good activity in both instances with the two fractions present. With use of an enzyme preparation from ox liver it was possible to establish that Fractions A and B contained thiolase and transferase, respectively. As previously reported (7), partially purified ox liver enzyme fractions were able to catalyze the synthesis of acetoacetate from acetyl CoA. This activity was not affected by the presence or absence of succinate and it was assumed that the endergonic condensation of 2 C_2 units in this case was facilitated by the exergonic deacylation of the presumed intermediate, acetoacetyl-S-CoA, through the agency of a deacylase. The acetoacetate-synthesizing activity of these fractions was largely destroyed by heating at 50° for a few minutes (Table III, Fraction L) but was restored by addition of the heart Fraction B, provided that succinate and succinyl CoA deacylase were present (*cf.* Table III). This indicated that Fraction L (apparently freed of acetoacetyl CoA deacylase by heating) contained thiolase and that the

heart Fraction B contained transferase. The similar behavior of the liver Fraction L and the heart Fraction A, shown by the data of Table III, indicated that the latter also contained thiolase.

It could further be shown that a product with the expected properties of acetoacetyl-S-CoA was formed on incubation of succinyl-S-CoA and acetoacetate with Fraction B but not with Fraction A or L. This product was converted to citrate, in the presence of either Fraction A or L (but not in its absence), CoA, oxalacetate, and crystalline condensing enzyme. This

TABLE III

Separation of Heart Transferase and Thiolase

The citrate assay system was identical to that of Table I except for enzyme fractions as indicated. The acetoacetate assay system contained (in micromoles) Tris-HCl buffer, pH 8.1, 150; KCl, 50; $MgCl_2$, 4; potassium L-cysteine, 20; lithium acetyl phosphate, 50; CoA, 0.1; transacetylase, 10 units (13); potassium succinate, 100; succinyl CoA deacylase (11), 1.1 mg. of protein. Other enzyme fractions as indicated. Final volume, 2.0 ml. The transacetylase was a partially purified preparation obtained from *Streptococcus hemolyticus* by an unpublished method of Korkes (see "Methods"). The preparation of the ox liver fraction (Fraction L) is described under "Methods." Fractions A and B (see the text) each contained P enzyme (11) in excess. Incubation, 60 minutes at 38°. The amount of protein used (in mg.) was for Fraction A, 4.8; Fraction B, 5.2; Fraction L, 5.5.

Enzyme fraction	Citrate formed	Acetoacetate formed
	μmoles	*μmoles*
A	0.39 (0.08)*	0.38
B	0.77 (0.03)	0.08
A + B	4.50 (0.07)	1.97
L		0.12 (0.11)†
L + A		0.40 (0.08)
L + B		1.50 (0.21)

* Values in parentheses obtained with succinate omitted from assay system.

† Values in parentheses obtained with succinate and succinyl CoA deacylase omitted from assay system.

reaction, which, as previously reported (16), leads to the net appearance of 1 mole of sulfhydryl per 2 moles of citrate formed, can be used for the quantitative determination of acetoacetyl CoA. These observations provided further support for the above conclusions regarding the nature of Fractions A, B, and L and for the mechanism of acetoacetate breakdown and synthesis represented by Reactions 1 and 2.

Optical Evidence for Transferase and Thiolase—The study of the properties of synthetic analogues of acetoacetyl-S-CoA by Lynen *et al.* (21) showed that *S*-acetoacetyl-*N*-acetylthioethanolamine (*S*-acetoacetyl-*N*-acetylcysteamine) has a characteristic absorption band at alkaline reactions with

a maximum at wave-length 303 mμ. This band is pH-dependent and may be attributed to the formation of the enolate ion $CH_3—\overset{O^-}{\underset{|}{C}}=CH—\overset{O}{\underset{\|}{C}}—S—R$ (18, 22). We have observed that acetoacetyl CoA (formed by the trans-

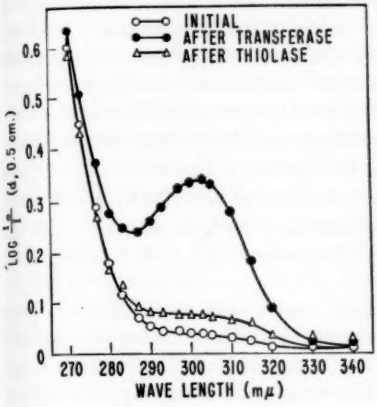

FIG. 1

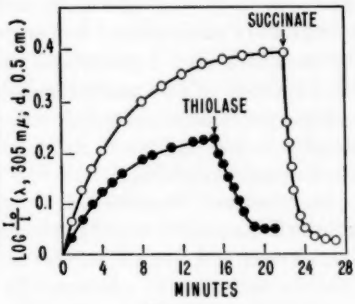

FIG. 2

FIG. 1. Spectral changes accompanying the enzymatic formation and breakdown of acetoacetyl-S-CoA at pH 8.1. Silica cells, d = 0.5 cm. Final volume, 1.5 ml.; temperature, 25°. ○, succinyl-S-CoA (~0.1 μmole). ●, after establishment of equilibrium on addition of acetoacetate (50 μmoles) and transferase (60 γ of protein). △, after further addition of CoA-SH (0.15 μmole) and thiolase (90 γ of protein). Tris-HCl buffer, pH 8.1 (100 μmoles), and $MgCl_2$ (8 μmoles) present in the reaction mixture. Acetoacetate and CoA-SH added to both blank and experimental cells. Mg^{++}, which is not required for these reactions, was added to increase the sensitivity of the assay (see the text).

FIG. 2. Optical demonstration of transferase and thiolase activities. Upper curve, transferase (17 γ of protein) added at zero time to a mixture of succinyl-S-CoA (~0.11 μmole) and acetoacetate (50 μmoles); succinate (40 μmoles) added at the arrow. Lower curve, transferase (17 γ of protein) added at zero time to a mixture of succinyl-S-CoA (~0.06 μmole) and acetoacetate (50 μmoles); CoA-SH (0.15 μmole) and thiolase (2.7 γ of protein) added at the arrow. Other details as in Fig. 1.

ferase reaction) has similar spectral properties and that the enol absorption band is markedly increased by Mg^{++} (16). The effect of Mg^{++} is presumably the result of metal chelation, since acetoacetic ester and β-diketones are known to be chelating agents (23). Thus, the formation and disappearance of acetoacetyl CoA through progress of Reaction 1 or 2 in either direction can be readily followed spectrophotometrically.

Fig. 1 shows the appearance of the enol band of *S*-acetoacetyl thio esters when synthetic succinyl CoA (24) is incubated, at pH 8.1, with acetoacetate

and a partially purified preparation of pig heart transferase free of thiolase. This demonstrates the formation of acetoacetyl CoA according to Reaction 1. Within the range 280 to 330 mμ the absorption band shown in Fig. 1 (*cf.* Fig. 3) agrees closely with the corresponding band of *S*-acetoacetyl-*N*-acetylthioethanolamine (*cf.* (18, 22)). It may also be seen that most of the absorption in this region disappears after the further addition of CoA and thiolase, since the equilibrium position of the thiolase reaction (Reaction 2) favors the formation of acetyl CoA. The thiolase was a partially purified preparation from pig heart free of transferase. In Fig. 2 the upper curve shows the increase in absorption at 305 mμ on adding transferase to a mixture of succinyl CoA and acetoacetate and the reversal of the reaction by succinate after equilibrium was established. The lower curve shows that addition of CoA and thiolase to a mixture of succinyl CoA and acetoacetate previously incubated with transferase causes a decrease in optical density at 305 mμ due to cleavage of the acetoacetyl CoA formed by the CoA transfer reaction.

Isolation and Properties of Acetoacetyl CoA—CoA was transferred from synthetic succinyl CoA (24) to acetoacetate, in the presence of pig heart transferase, and the acetoacetyl CoA was isolated as a crude barium salt. The reaction mixture contained the following components (in micromoles): Tris-HCl buffer, pH 7.5, 1000; $MgCl_2$, 80; succinyl CoA, about 20; potassium acetoacetate, 1000; and heart Fraction B, prepared as described above, with 33 mg. of protein. Following the incubation for 25 minutes at 25°, the mixture was chilled and the protein was precipitated with ice-cold trichloroacetic acid, in a final concentration of 2 per cent, and removed by centrifugation. The supernatant solution (22 ml.), which contained 0.6 μmole of acetyl CoA and 5.1 μmoles of acetoacetyl CoA,[3] was adjusted to pH 7.2 with 2.0 N KOH; this was followed by the addition of 4.0 ml. of 1.0 M barium chloride and 90 ml. of ice-cold 95 per cent ethanol. The solution was adjusted to pH 7.0 and kept at 0° for 45 minutes. The precipitate was collected by centrifugation, washed twice with 75 per cent ethanol, and dried in vacuum over calcium chloride. The yield of crude barium salt was 110 mg. For assay it was converted to the soluble potassium salt. 110 mg. of barium salt contained 2.6 μmoles of acetoacetyl CoA or about 3 mg. of its dibarium salt. Thus, the yield was about 51 per cent and the purity 3 per cent.

The acetoacetyl CoA could be separated from the succinyl CoA and acetoacetate in the crude preparation by paper chromatography. The solution of the potassium salt was chromatographed on Whatman No. 1 filter paper with ethanol-sodium acetate (13) as solvent for 4 hours at 24°. The aceto-

[3] Determined from the citrate formed, in the presence of oxalacetate and crystalline condensing enzyme, before and after addition of CoA and thiolase.

acetyl CoA band (R_F 0.52) which overlapped the oxidized CoA band (R_F 0.50) gave the following color reactions: (*a*) The direct nitroprusside reaction with weak alkali was negative, but after spraying with strong alkali followed by nitroprusside an orange color appeared owing to the release of acetoacetate as well as CoA-SH (CoA-SH on paper normally gives a purple-pink color with nitroprusside); (*b*) after spraying with alkali followed by neutralization with acid, ferric chloride spray also caused an orange color to appear. Free acetoacetate, which also gave an orange color with both alkaline nitroprusside and ferric chloride, migrated much more rapidly (R_F 0.75) than the thio ester. In another run, a larger amount of the crude potassium salt was placed along the edge of Whatman No. 1 filter paper and chromatographed as above, but for 5 hours at 3°. The acetoacetyl CoA band (R_F 0.42) was cut out and eluted with water at 3°. By enzymatic and optical assay the eluate (5.0 ml.) contained 0.77 μmole of acetoacetyl CoA and less than 0.1 μmole of acetyl CoA. The ultraviolet absorption spectrum of the eluate in 0.067 M Tris-HCl buffer, pH 8.1, showed an absorption band in the wave-length range 290 to 320 mμ, with a maximum at 305 mμ, and a second absorption band with a peak at 260 mμ. The latter band, due to the CoA moiety, was independent of the pH, but at neutral or acid pH there was no absorption at 305 mμ. The absorption at 305 mμ (pH 8.1) was increased over 2-fold by addition of 5×10^{-3} M $MgCl_2$. These properties characterized the compound as acetoacetyl CoA. Addition of neutral hydroxylamine (100 μmoles, pH 8.0) caused the absorption at 305 mμ to disappear, the absorption at 260 mμ remaining unchanged. However, the product is not a hydroxamic acid, since it gives no color with ferric chloride. Although the reaction of acetoacetic ester with hydroxylamine is followed by ring closure to form methyl isooxazolone (25) with a characteristic absorption spectrum,[4] no such spectrum was obtained following reaction of acetoacetyl CoA with hydroxylamine. Addition of thiolase and CoA caused a disappearance of the light absorption at 305 mμ and a concomitant increase in absorption at wave-length 240 mμ. This is the result of the formation of a second thio ester bond (*cf.* Reaction 2) since thio esters have an absorption band in the 230 to 240 mμ region (26, 27). The presence of acetoacetyl CoA in the eluate was further confirmed by the fact that it brought about oxidation of DPNH, in the presence of liver or heart fractions containing β-hydroxybutyryl CoA dehydrogenase, and the fact that the light absorption at 305 mμ (pH 8.1) of a mixture of eluate and succinate was decreased by addition of transferase (Reaction 1 from left to right).

The ultraviolet absorption spectrum of acetoacetyl CoA, in the absence and presence of Mg^{++}, is presented in Fig. 3, *A*. Fig. 3, *B* shows the differ-

[4] S. Korkes, personal communication.

ence spectrum of intact *versus* hydrolyzed acetoacetyl CoA, whereby the spectrum of the CoA moiety of the thio ester is eliminated, without and with Mg^{++}. As in the case of *S*-acetoacetyl-*N*-acetylthioethanolamine (22) the enolate ion band with a peak at 302 mμ, found at pH 8.1, is absent at pH 6.1. In the latter case only the thio ester band with a maximum at

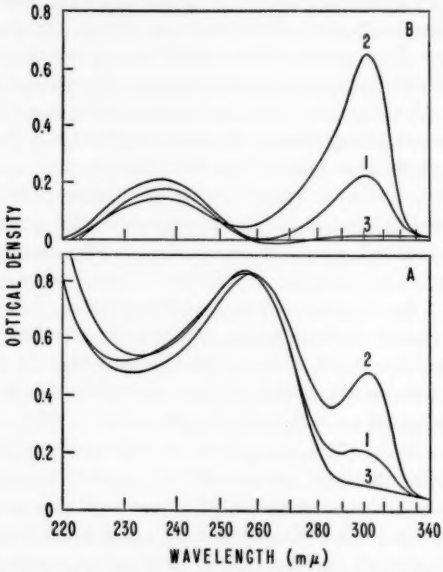

FIG. 3. Ultraviolet absorption spectrum of acetoacetyl-S-CoA. *A*, the blank cell contained water; the experimental cell acetoacetyl CoA (0.03 μmole per ml.). *B*, difference spectrum of intact *versus* hydrolyzed acetoacetyl CoA. The blank cell contained a solution in which acetoacetyl CoA (0.05 μmole per ml.) was previously hydrolyzed by incubation at pH 8.1 and 45° for 2.5 hours; the experimental cell contained acetoacetyl CoA (0.05 μmole per ml.). Curves 1, pH 8.1 (0.1 M Tris-HCl buffer), no $MgCl_2$; Curves 2, pH 8.1, 5×10^{-3} M $MgCl_2$; Curves 3, pH 6.1 (0.1 M acetate buffer), 5×10^{-3} M $MgCl_2$. Silica cells; $d = 1.0$ cm. The acetoacetyl CoA was prepared by reaction of CoA-SH with diketene (22). The spectra were obtained with a Beckman model DK 2 recording spectrophotometer.

237 mμ (22, 26, 27) is present (Fig. 3, *B*). The molecular extinction coefficient at wave-length 305 mμ of enzymatically generated acetoacetyl CoA was determined as follows: Tris-HCl buffer (100 μmoles), pH 8.1, $MgCl_2$ (8 μmoles), succinyl CoA (about 1 μmole), potassium acetoacetate (100 μmoles), and heart transferase were mixed in a silica cell ($d = 0.5$ cm., final volume = 1.5 ml.) and the optical density at 305 mμ, resulting from acetoacetyl CoA formation, was read at minute intervals in a Beckman

spectrophotometer against a reference cell containing the same components except succinyl CoA. At equilibrium, when the optical density change was 0.960, the solution in the experimental cell was deproteinized with ice-cold trichloroacetic acid (final concentration, 2 per cent), the protein precipitate was removed by centrifugation, and the supernatant fluid was neutralized. By enzymatic assay with thiolase and citrate condensing enzyme, in the presence of CoA and oxalacetate, the solution was found to contain 0.204 μmole of acetoacetyl CoA, corresponding to a concentration of 1.36×10^{-4} M in the 1.5 ml. of reaction mixture originally present in the experimental cell. Thus, at pH 8.1 and in the presence of 5.3×10^{-3} M Mg^{++}, the molecular extinction coefficient at 305 mμ of acetoacetyl CoA is 14,000. It should be emphasized that this is not the actual value of the extinction coefficient of the enolate-Mg chelate, but is calculated on the basis of the acetoacetyl CoA concentration.

Methods—The pig heart 20–40 ethanol fraction, used in the experiment of Table I, was prepared from the 40–60 ammonium sulfate fraction described above by precipitation with ethanol between the limits of 20 and 40 per cent concentration (by volume) at −15° and pH 7.4. The conditions, solution of the precipitate, and dialysis were otherwise the same as those for the ethanol fractions described under "Fractionation of enzymes."

The ox liver Fraction L, used in the experiments of Table III, was prepared as follows: Ox livers were frozen immediately after slaughtering. The frozen liver was thawed and cut into small pieces. It was extracted in a Waring blendor, 150 gm. batches at a time, with 100 ml. of 0.2 M potassium bicarbonate containing 0.005 M L-cysteine (pH 8.5) for 3 to 4 minutes at 3–4°. The homogeneous suspension obtained at this point was diluted with a further 200 ml. of the bicarbonate solution and blended for 1 more minute. The suspension was strained through two layers of cheese-cloth and centrifuged for 20 minutes at 3–4° and 3000 r.p.m. in the large head of the International centrifuge. The large residue obtained was discarded. The supernatant fluid, containing about 50 mg. of protein per ml., was diluted with 2 volumes of the bicarbonate solution used for extraction and fractionated with ammonium sulfate. Solid ammonium sulfate was added at 0° with mechanical stirring. The fraction between 0 and 0.3 saturation was discarded. The supernatant solution was brought to 0.6 ammonium sulfate saturation at 0° and, after stirring for 20 minutes, the precipitate was collected by centrifugation in the Servall angle centrifuge in the cold room, dissolved in 0.02 M potassium phosphate buffer, pH 7.0, containing 0.005 M L-cysteine, and dialyzed overnight against the same buffer at 3–4°. The clear reddish solution contained 40 to 50 mg. of protein per ml. This solution was brought to pH 5.5 with 1.0 N acetic acid; the precipitate was removed by centrifugation and discarded, and the supernatant fluid was

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either dialyzed overnight against 0.02 M potassium phosphate buffer, pH 7.0, or adjusted to pH 7.0 with 1.0 N KOH. The neutralized solution was heated to 50° for 5 to 10 minutes with constant stirring, chilled, and centrifuged. The precipitate, which was usually small, was removed by centrifugation and discarded. Under the assay conditions of Table III, an amount of *unheated* Fraction L containing 5.5 mg. of protein catalyzed the formation of about 1.5 μmoles of acetoacetate in the absence of protein fractions other than transacetylase. After heating, the same fraction catalyzed the formation of only 0.12 μmole of acetoacetate (*cf.* Table III).

The transacetylase used in the acetoacetate synthesis assay system was prepared by the following procedure:[5] *Streptococcus hemolyticus* (type C) was obtained[6] in the form of a filter cake containing over 90 per cent by volume of Celite. It was kept frozen. The material was thawed and suspended in 0.1 M potassium phosphate buffer, pH 7.4, to make a fluid paste. After standing for about 20 minutes, with occasional stirring, the mixture was placed in a Waring blendor and stirred for 7 minutes at 3–4°. The suspension was centrifuged for 20 minutes at 13,000 r.p.m. in the Servall angle centrifuge in the cold room and the sediment was discarded. The supernatant fluid was brought to 70 per cent saturation with solid ammonium sulfate at 0° and the precipitate was stored at −18°. At this stage the preparation contained transacetylase (13) and acetokinase (28). The ammonium sulfate paste was diluted with 1 volume of water and acidified to pH 4.0 with 1.0 N hydrochloric acid; the precipitate was discarded. The supernatant solution was brought to 70 per cent saturation with solid ammonium sulfate and centrifuged; the precipitate was dissolved in 0.1 M dipotassium phosphate and dialyzed overnight against 0.02 M potassium phosphate buffer, pH 7.4, containing 0.001 M L-cysteine. This preparation was free of acetokinase and contained about 150 units of transacetylase (13) per ml.; its protein concentration was about 19 mg. per ml.

Crystalline condensing enzyme was prepared as described by Ochoa *et al.* (29). Succinyl-S-CoA was prepared synthetically according to Simon and Shemin (24). Citric acid was determined by the method of Natelson *et al.* (30) with some modifications. Acetoacetic acid was determined by the method of Greenberg and Lester (31), as modified by Barkulis and Lehninger (32), on trichloroacetic acid filtrates. In the presence of oxalacetate (*cf.* Table I) acetoacetate was determined by the aniline acetate method after decarboxylation of residual oxalacetate by addition of Cu^{++} according to the procedure of Hunter and Leloir (5). Protein was determined by the

[5] S. Korkes, unpublished. We are indebted to Dr. Korkes for permission to describe this procedure.

[6] From the Lederle Laboratories Division, American Cyanamid Company, through the courtesy of Mr. F. Ablondie.

method of Gornall *et al.* (33) or by measurement of the light absorption at wave-lengths 280 and 260 mμ according to Warburg and Christian (34).

SUMMARY

1. The cleavage of β-keto fatty acids by soluble enzyme preparations from animal tissues requires preliminary activation to the corresponding CoA thio ester derivatives. In heart muscle these derivatives are formed, in the case of acetoacetate, β-ketovalerate, and β-ketocaproate, by transfer of CoA from succinyl CoA.

2. Cleavage of the activated β-keto acids yields acetyl CoA and the acyl CoA derivative shorter by 2 carbon atoms than the parent β-keto acid.

3. The separation and some properties of two enzymes from pig heart extracts catalyzing the CoA transfer (transferase) and cleavage (thiolase) reactions are described. Reversibility of these reactions is responsible for the synthesis of acetoacetate from acetyl CoA in the presence of the two enzymes and succinate.

4. The isolation and characterization of acetoacetyl-S-CoA, formed by the enzymatic transfer of CoA from succinyl CoA to acetoacetate, are described.

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ENZYMES OF FATTY ACID METABOLISM

IV. PREPARATION AND PROPERTIES OF COENZYME A TRANSFERASE*

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Evidence for the reversible enzymatic transfer of CoA[1] from succinyl-S-CoA to acetoacetate according to Reaction 1 has been presented in Paper III (1). The enzyme catalyzing Reaction 1 has been named succinyl-β-

(1) $\text{Succinyl-S-CoA}^- + \text{acetoacetate}^- \rightleftharpoons \text{acetoacetyl-S-CoA}^- + \text{succinate}^= + H^+$

ketoacyl-S-CoA transferase (2) and will be referred to simply as CoA transferase or transferase.[2] The first enzyme of this type was described by Stadtman (3) in *Clostridium kluyveri* extracts and named CoA transphorase. It catalyzes the reversible transfer of CoA from acetyl-S-CoA to C_3 to C_8 monocarboxylic saturated fatty acids, vinylacetic and lactic acids, but not to β-keto acids. This paper describes the preparation and properties of highly purified CoA transferase, including substrate specificity and affinity, pH dependence, and equilibrium constants. The distribution of the enzyme and its significance in cardiac metabolism are discussed. A preliminary account of this work has appeared (4).

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[1] The abbreviations employed in Paper III (1) are adhered to. In addition, optical density ($\log I_0/I$) is abbreviated as E (extinction). ΔE_{310} stands for change in optical density at wave-length 310 mμ. EDTA = ethylenediaminetetraacetic acid, FAD = flavin-adenine dinucleotide.

[2] A Committee on Nomenclature, 2nd International Conference on Biochemical Problems of Lipides, Ghent, Belgium, July, 1955, has proposed the systematic name acetoacetylsuccinic thiophorase for CoA transferase.

Enzyme Assay and Unit

The direct optical assay is based on the finding of Lynen *et al.* (5) that, at alkaline pH, acetoacetyl thio esters, *e.g.* *S*-acetoacetyl-*N*-acetylthioethanolamine (6) and acetoacetyl-S-CoA (1, 6), exhibit a characteristic absorption band in the range 280 to 320 mμ, with a maximum at 303 mμ. This absorption is attributed to formation of an enolate ion and is increased by a decrease in H^+ ion concentration. Stern *et al.* (4) observed that this enolate absorption of acetoacetyl CoA is markedly increased by Mg^{++} ions, probably through formation of a chelate compound. The presence of Mg^{++} ions, which are not required for CoA transferase activity, increases the sensitivity of the optical assay. Succinyl CoA has no significant absorption in the enolate ion region. It is important to note that the enolate ion absorption is abolished by 10^{-4} M Hg ions (7) and by 6×10^{-3} M L-cysteine, but not by glutathione[3] or thioglycolate.[4]

The optical assay is performed at wave-length 310 mμ with a Beckman spectrophotometer in 2.0 ml. silica cells of 0.5 cm. light path. To the experimental cells are added 0.10 ml. of 1.0 M Tris-HCl buffer, pH 8.1, 0.01 ml. of 0.8 M $MgCl_2$, 0.10 ml. of 1.0 M potassium acetoacetate, 0.20 ml. of 0.0018 M succinyl CoA, enzyme, and water to a final volume of 1.50 ml. The reference cell contains all the assay components except succinyl CoA. The enzyme is diluted with 0.02 M potassium phosphate buffer, pH 7.4, so that 0.01 ml. contains 3 to 40 γ of protein, depending on its activity. The reaction is started by addition of enzyme and the increase in optical density at 310 mμ is recorded at 0.5 minute intervals at 25°. The ΔE_{310} from 0.5 to 1.0 minute after addition of enzyme is used to calculate its activity.

1 unit of enzyme is defined as the amount which causes an initial rate of increase in optical density ($+\Delta E_{310}$) of 0.01 per minute under the above conditions. As illustrated in Fig. 1, the initial rate of $+\Delta E_{310}$ is proportional to enzyme concentration, provided it does not exceed 0.04 per minute. The initial rate is not maintained beyond 2 to 3 minutes, probably because of the unfavorable equilibrium position when the reaction is measured in this direction. The specific activity is expressed as units per mg. of protein. Protein is determined spectrophotometrically by the method of Warburg and Christian (8). The molecular extinction coefficient (ϵ_{310}) of acetoacetyl CoA under the assay conditions (pH 8.1, Mg^{++} present) is 11,900. Therefore, 1 unit corresponds to the formation of 0.0025 μmole of acetoacetyl CoA. It should be noted that the direct assay can be modified to measure the favored enzymatic conversion of acetoacetyl CoA to succinyl CoA (see below).

[3] F. Lynen, personal communication.
[4] J. R. Stern, unpublished observations.

Assay of Crude Tissue Extracts—The direct optical method of assay can only be employed with enzyme fractions which are free of acetoacetyl thiolase. Thiolase is removed in Steps 3 and 4 of the purification procedure. With cruder fractions and tissue extracts, the transferase can be assayed optically by coupling with thiolase and trapping the acetyl-S-CoA thus generated with oxalacetate, with use of the malic dehydrogenase-condensing enzyme system (9, 10) or with *p*-nitroaniline and the aromatic

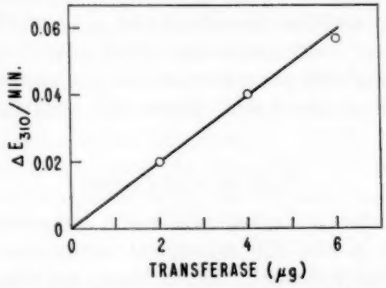

FIG. 1. Optical assay of transferase. The assay conditions are given in the text. Transferase, specific activity 900, was used.

amine-acetylating enzyme of pigeon liver (11, 12). The sequence of reactions is as follows:

(2) Succinyl-S-CoA + acetoacetate $\rightleftharpoons$ acetoactyl-S-CoA + succinate

(3) Acetoacetyl-S-CoA + HS-CoA $\rightleftharpoons$ 2 acetyl-S-CoA

(4a) 2 Malate + 2DPN$^+$ + 2 acetyl-S-CoA $\rightleftharpoons$
2 citrate + 2HS-CoA + 2DPNH + 2H$^+$

(4b) 2 Acetyl-S-CoA + 2 *p*-nitroaniline $\rightarrow$ 2 *p*-nitroacetanilide

The reduction of DPN$^+$ can be followed at wave-length 340 mμ and the acetylation of *p*-nitroaniline at 420 mμ (12).

The components of the DPN$^+$ reduction assay (in micromoles) are Tris-HCl buffer, pH 8.1, 100; $MgCl_2$, 8; potassium acetoacetate, 100; CoA, 0.2; neutralized GSH, 10; DPN$^+$, 0.3; potassium L-malate, 10; succinyl CoA, 0.35; purified thiolase, 200 units (specific activity 15,000); purified malic dehydrogenase, 2 γ (specific activity 62,000); crystalline condensing enzyme, 16 γ (specific activity 330); and CoA transferase fraction, 3 to 250 γ. The reaction is started by addition of succinyl CoA. 1 unit of transferase is defined as the amount which causes an initial rate of increase in optical density at 340 mμ of 0.01 per minute at 25°. 1 unit corresponds to the formation of 0.0048 μmole of DPNH or 0.0024 μmole of acetoacetyl CoA.

If the extract is contaminated with DPNH oxidase (the use of cyanide as inhibitor may be tried (10)), the *p*-nitroaniline assay should be used. The components of the latter (in micromoles) are Tris-HCl buffer, pH 8.1, 100; $MgCl_2$, 8; potassium acetoacetate, 100; CoA, 0.2; neutralized GSH, 10; succinyl CoA, 0.35; *p*-nitroaniline, 0.3; purified pigeon liver acetylating enzyme, 0.3 mg. (specific activity 150 to 200); and transferase fraction. Purified thiolase (200 units) must also be added after Step 2 if not already supplied in excess by the acetylating enzyme fraction employed. The reaction is started by addition of succinyl CoA. 1 unit of transferase is defined as the amount which causes an initial rate of decrease in optical density at 420 mμ of 0.01 per minute at 25°. 1 unit corresponds to the formation of 0.0050 μmole of *p*-nitroacetanilide or 0.0025 μmole of acetoacetyl CoA.

Purification of Enzyme

Step 1. Preparation of Extract—Pig hearts, removed immediately after death, are packed in ice. All subsequent operations are performed at 0–3° unless otherwise indicated. Thirty hearts are trimmed of fat, blood clots, and connective tissue and passed twice through an electric mincer. The minced heart (about 4.5 kilos) is washed five times with 5 volumes of cold tap water in a tall glass cylinder (capacity 37 liters). Cylindrical blocks of ice (volume about 800 ml.) are used to keep the suspension cold. The washed mince is then passed through a large table type Büchner filter and dried as far as possible by suction. 170 gm. portions of the washed mince are placed in a Waring blendor and 1.5 volumes (255 ml.) of 0.05 M potassium phosphate buffer, pH 7.4, containing 0.2 M KCl are added. The suspension is stirred for 5 minutes at two-thirds the maximal speed; then another 1.5 volumes of buffer mixture are added and the stirring is continued for another 5 minutes at one-third maximal speed. The suspension is passed through two layers of cheese-cloth and centrifuged in a Servall angle centrifuge at 13,000 × *g* for 7 minutes. The deep pink supernatant fluid is passed through ten layers of cheese-cloth to remove floating fat particles. About 10 liters of extract are obtained.

Step 2. Fractionation with Ammonium Sulfate—5 liter portions of the extract are brought to 35 per cent saturation with powdered ammonium sulfate (245 gm. per liter). The salt is added slowly over a period of 20 minutes with mechanical stirring. The mixture is stirred for a further 20 minutes and centrifuged at 13,000 × *g* for 15 minutes. The precipitate is discarded. The supernatant fluid is brought to 65 per cent saturation by adding 210 gm. of ammonium sulfate for each liter of original solution in the manner indicated above. The precipitate is dissolved in 500 ml. of 0.017 M potassium phosphate buffer, pH 6.8, and dialyzed against 20 liters of the same buffer for 16 hours.

Step 3. Fractionation with Acetone—The 35–65 ammonium sulfate fraction is made 0.067 M with respect to potassium phosphate buffer, pH 6.8. Cold acetone (−10°) is then added to a concentration of 40.5 per cent by volume with mechanical stirring, the temperature of the mixture being gradually lowered to −5° during the 30 to 40 minute period required for the addition. Stirring is continued for another 15 minutes at −5°. The mixture is centrifuged at 1100 × *g* for 30 minutes at a temperature setting low enough to insure that the temperature of the supernatant solution will be −5° to −6°. The precipitate is dissolved in 150 ml. of 0.017 M potassium phosphate buffer, pH 7.4, and the solution is dialyzed overnight against 20 liters of the same buffer. This 0–40.5 fraction is used for the purification of thiolase. The cloudy supernatant fluid is brought to 57 per cent acetone concentration by volume at −9° to −10° and, after centrifugation at this temperature, the precipitate is dissolved in 150 ml. of 0.017 M potassium phosphate buffer, pH 7.4, and the solution is dialyzed overnight against 20 liters of the same buffer.

In practice, stocks of the 0–40.5 and 40.5–57 acetone fractions are accumulated as starting material for the further purification of thiolase and transferase, respectively, and kept frozen. Transferase is remarkably stable at all stages of purification and can be kept in the deep freeze for many months with little or no loss of activity.

Step 4. Heat and Acid Treatment—The 40.5–57 acetone fraction is diluted with 0.017 M potassium phosphate buffer, pH 7.4, to bring the protein concentration to 10 mg. per ml. 300 ml. portions, in a 1 liter glass beaker, are placed in a bath at 55° and stirred constantly. The temperature of the solution is allowed to rise to 50° during an interval of 9 minutes, the bath temperature falling to about the same reading. The solution is maintained at 50° for another 6 minutes and then placed in an ice bath. The precipitate is removed by centrifugation at 13,000 × *g* for 20 minutes and discarded. About 65 ml. of 0.1 N acetic acid are added rapidly with mechanical stirring to the supernatant fluid until the pH falls to 5.8. The precipitate is removed by centrifugation at 13,000 × *g* for 5 minutes and discarded.

Step 5. Adsorption on Alumina Gel[5]—The solution from Step 4 is quickly adjusted to pH 6.65 by addition of about 4 ml. of 1.0 M potassium bicarbonate. Alumina Cγ gel (dry weight, 13 mg. per ml.) is added to make a final concentration of 30 per cent by volume and the mixture is stirred for 10 minutes at 0°. The gel is separated by centrifugation and the supernatant fluid is discarded. The gel is suspended in a volume of cold 0.1 M potassium phosphate buffer, pH 7.4, equal to that of the starting solution and stirred continuously for 10 minutes at 0°. After centrifuga-

[5] We are indebted to Dr. S. Kaufman and Dr. C. Gilvarg, who first worked out this step for the purification of P enzyme from pig heart.

tion, this eluate (718 ml. containing 1750 mg. of protein, specific activity 4) is discarded and the gel is again eluted by suspending it in a similar volume of 0.2 M potassium phosphate buffer, pH 8.0 (20°), and stirring at 20° for 20 minutes. After removal of gel by centrifugation, the eluate at pH 8, containing about 1 mg. of protein per ml., is concentrated by addition of solid ammonium sulfate to 85 per cent saturation, centrifuging, and dissolving the precipitate in a volume of 0.017 M Tris buffer, pH 7.5, equal to 5 per cent of that of the eluate. This fraction is dialyzed overnight against the same buffer to remove residual inorganic phosphate as well as ammonium sulfate.

Step 6. Ethanol Fractionation in Presence of Zinc—The dialyzed eluate at pH 8 is diluted to a protein concentration of about 5 mg. per ml. with 0.017 M Tris buffer, pH 7.5, and 0.025 volume of 1.0 M potassium succinate, pH 6.2, is added. 0.25 volume of 0.1 M zinc acetate is added slowly with stirring at 0°. After 5 minutes the mixture is centrifuged and the precipitate (89.4 mg. of protein, specific activity 4) is discarded. Cold absolute ethanol is added to the clear supernatant fluid at 0° to a concentration of 15 per cent by volume and, after stirring for 10 minutes, the mixture is centrifuged and the red brown precipitate (262 mg. of protein, specific activity 12) is discarded. The supernatant solution is brought to 35 volumes per cent ethanol concentration, stirred for 10 minutes at 0°, and then centrifuged. The yellowish precipitate is dissolved in 10 ml. of 0.1 M potassium phosphate buffer, pH 7.4, containing 0.01 M potassium EDTA and 0.1 per cent GSH, and the solution is dialyzed overnight against 0.017 M potassium phosphate buffer, pH 7.4. Any turbidity which may appear is removed by centrifugation, leaving a clear yellow solution.

Step 7. Fractionation with Ammonium Sulfate—The procedure is that of Step 2. The 15–35 zinc-ethanol fraction is diluted to a protein concentration of 5 mg. per ml. with 0.017 M potassium phosphate buffer, pH 7.4, and then brought to 45 per cent saturation with ammonium sulfate. The precipitate (5.6 mg. of protein, specific activity 168) is discarded and the supernatant fluid obtained after centrifugation is brought to 58 per cent saturation with ammonium sulfate. This precipitate is collected and dissolved in a few ml. of 0.017 M potassium phosphate buffer, pH 7.4, to give a clear yellow solution which is dialyzed overnight against the same buffer. Data on the purification of the enzyme are summarized in Table I.

Properties of Enzyme

Purity—The 45–58 ammonium sulfate fraction is practically the most active transferase preparation which has been obtained. Its specific activity falls in the range 1000 ± 50. This fraction, concentrated solutions of which were visually yellow, contained a fluorescent flavoprotein. Ul-

tracentrifugal analysis[6] showed that it contained two protein components. The major component, comprising about 80 per cent of the total protein, was the transferase (see below). The minor component, which sedimented somewhat less rapidly and represented about 20 per cent of the protein, was the flavoprotein. The sedimentation constant ($s_{20,w}$) of the CoA transferase component was 5.08×10^{-13} per second for a 1.22 per cent solution in 0.02 M potassium phosphate buffer, pH 7.4. Assuming a partial specific volume of 0.75 and a frictional ratio ($f:f_0$) between 1.0 and 1.8, the molecular weight would be from 46,000 to 112,000.

TABLE I

Purification of CoA Transferase

Step No.	Volume	Units	Protein	Specific activity*	Yield
	ml.		*mg.*	*units per mg. protein*	*per cent*
1. Phosphate extract†	10,500	181,000	151,000	1.2‡	100
2. $(NH_4)_2SO_4$ (35–65)	1,240	156,000	25,900	6.0§	86
3. Acetone ppt. (40.5–57)	474	86,700	8,250	10.5§	48
4. Acid supernatant fluid	723	68,300	4,880	14.0‖	38
5. Alumina eluate¶	46	63,400	576	110‖	35
6. Zinc-ethanol (15–35)	15.6	42,700	95.1	449‖	24
7. $(NH_4)_2SO_4$ (45–58)	1.8	34,300	34.3	1000‖	18

* Calculated in units of direct assay.
† From 4.5 kilos of heart.
‡ *p*-Nitroaniline assay.
§ DPN^+ reduction assay.
‖ Direct assay.
¶ After concentration.

Identification of Components—Paper electrophoresis[7] confirmed the presence of two protein components in the 45–58 ammonium sulfate fraction. The migration of the flavoprotein could be readily followed by its fluorescence in ultraviolet light. In a typical experiment, 0.42 mg. of purified enzyme (specific activity 1000) was placed on a paper strip (Whatman No. 1) and run with a current strength of 1.0 ma., a solvent of 0.05 M potassium phosphate buffer, pH 7.9, and a temperature of 3°. After 20 hours, the position of the components was determined on a separate paper strip containing 0.11 mg. of the enzyme and treated identically. On both strips the flavoprotein had migrated 5.4 ± 0.2 cm. toward the anode. Staining the

[6] We are indebted to Dr. I. B. Wilson, College of Physicians and Surgeons, Columbia University, for the ultracentrifuge runs.

[7] We are grateful to Dr. C. V. Tondo and Dr. K. G. Stern, Polytechnic Institute of Brooklyn, for use of their facilities.

control strip with Amido Schwarz 10B revealed, in addition, a second protein component, which had migrated 4.0 ± 0.4 cm. toward the anode. The corresponding areas of the experimental strip were eluted with 1.0 ml. of 0.1 M potassium phosphate buffer, pH 7.4. The eluted proteins representing 69 per cent recovery were assayed enzymatically. The slower moving protein gave a positive transferase assay. Its specific activity of 83 indicated considerable inactivation of the enzyme during the electrophoretic run. The flavoprotein gave a negative assay for transferase and a positive diaphorase test (see below). When subjected to paper electrophoresis in 0.05 M potassium phosphate buffer, pH 6.0, the flavoprotein migrated toward the anode and the transferase toward the cathode. This suggested that the isoelectric point of the flavoprotein was below, and that of transferase above, pH 6. Densitometer analysis of the stained paper electrograms gave a calculated 77 per cent of the total protein as transferase.

Nature of Flavoprotein—The absorption spectrum of the solution of the 45–58 ammonium sulfate fraction was typical of a flavoprotein and showed three absorption bands with maxima at 278, 355, and 455 mμ. The prosthetic group of the flavoprotein could be resolved by heat denaturation of the protein (pH 7.4), but not by acidification to pH 2.3 in 45 per cent ammonium sulfate solution (12). It was identified as flavin-adenine dinucleotide by its reactivity with D-amino acid oxidase apoprotein (13). The native flavoprotein in this fraction gave negative tests for diphosphopyridine nucleotide- and triphosphopyridine nucleotide-linked cytochrome *c* reductases, D- and L-amino acid oxidases, and fumarate reductase. It possessed fairly active diaphorase activity and, in an optical test system (d = 0.5 cm.) consisting of 0.067 M Tris-HCl buffer, pH 8.5, 2×10^{-4} M DPNH, and 0.9×10^{-4} M 2,6-dichlorophenolindophenol in a volume of 1.50 ml., caused a $-\Delta E_{620}$ of 0.8 per mg. of total protein. Under the same conditions the flavoprotein component isolated by paper electrophoresis causes a $-\Delta E_{620}$ of 1.0 per mg. of protein, suggesting some inactivation on electrophoresis. While several of its properties, fluorescence, tightly bound FAD, and absorption spectrum, paralleled those of Straub's heart diaphorase (14), no further attempt was made to characterize this flavoprotein enzyme.

Removal of Flavoprotein—The flavoprotein can be largely, but not entirely, removed from the 45–58 ammonium sulfate fraction (2.0 mg. of protein per ml.) by adsorption on 0.12 volume of alumina Cγ gel at pH 6.0. Higher concentrations of gel result in significant adsorption of the transferase. The specific activity of the CoA transferase in the gel supernatant fluid, which shows very slight fluorescence under ultraviolet light, is thereby increased about 20 per cent. The flavoprotein itself is readily eluted from the alumina Cγ with 0.2 M potassium phosphate buffer, pH

8.0. Assuming that the flavoprotein contains one flavin group per molecule and that the molecular extinction coefficient of flavoproteins at wavelength 455 mμ (ϵ_{455}) is 1040 (15), it can be calculated that its minimal molecular weight is 75,000.

Turnover Number—The specific activity of the purest transferase, which is obtained after alumina Cγ treatment and represents practically pure enzyme, falls in the range 1200 ± 50. Assuming that the pure CoA transferase would have a specific activity of 1300, it would catalyze the transfer of 325 moles of CoA per minute from succinyl-S-CoA to acetoacetate per 100,000 gm. of enzyme at pH 8.1 and 25°. As shown below, the enzyme catalyzes the transfer of CoA from acetoacetyl-S-CoA to succinate at a rate about 25 times faster than that of the reverse reaction cor-

TABLE II

Substrate Specificity of Purified Transferase

The specific activity was determined under standard assay conditions with 100 μmoles of β-keto acid. Activity of acetoacetate taken as 100.

β-Keto acid	Specific activity	Relative activity	Relative activity*
Acetoacetate	702	100	100
β-Ketovalerate	491	70	61
β-Ketoisocaproate	403	57	10
β-Ketocaproate	228	32	34
β-Ketooctanoate	0	0	0
β-Ketoadipate	0	0	0

* Calculated from measurements (18) of the rate of citrate synthesis by a crude ethanol fraction of pig heart which contained CoA transferase (specific activity ~10) and excess thiolase and condensing enzyme.

responding to a turnover number of 8000. It is noteworthy that the turnover number of CoA transferase is of the same order of magnitude as that of crystalline citric condensing enzyme (turnover number 5000) also prepared from pig heart (16).

The purified transferase (specific activity 1140) is free of thiolase, crotonase, β-hydroxybutyryl-S-CoA dehydrogenase, condensing enzyme, aconitase, the P enzyme system, and succinyl-S-CoA deacylase. It contains traces of fumarase hydrating 0.44 μmole of fumarate per mg. per minute at pH 8.1 and 25°. Since the turnover number of crystalline fumarase is about 100,000 (17), this transferase preparation contained about 0.09 per cent fumarase. On the basis of the maximal specific activity, it can be calculated that 0.09 per cent of the protein in the initial heart extract is CoA transferase.

Specificity—As shown in Table II, the purified CoA transferase catalyzes the transfer of CoA from succinyl-S-CoA to acetoacetate, β-ketovalerate,

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β-ketoisocaproate, and β-ketocaproate in order of decreasing activity. It is seen that, β-ketoisocaproate excepted, the relative activity of the β-keto acids measured by direct optical assay with the purified enzyme agrees well with that reported earlier (17) for crude heart fractions by using a multienzyme assay based on citrate synthesis. It appears that in crude fractions the cleavage of β-ketoisocaproyl-S-CoA by thiolase was the rate-limiting step and was considerably slower than the cleavage of β-ketocaproyl-S-CoA. The purified transferase also transfers CoA from succinyl-S-CoA to α-methylacetoacetate[8] but not to β-ketooctanoate, β-ketoadipate, benzoylacetate, crotonate, β-methylcrotonate, and saturated fatty acids. It catalyzes CoA transfer from acetoacetyl-S-CoA to succinate, but not to malonate, glutarate, butyrate, β-hydroxybutyrate, crotonate, or malate. It does not catalyze CoA transfer from crotonyl-S-CoA to succinate, nor from maleyl-S-CoA to acetoacetate. Thus the transferase is strictly specific for the succinyl moiety in regard to chain length, substitution, and introduction of a double bond. It is less specific for the β-keto acyl moiety which may vary in chain length from C_4 to C_6, and which may be substituted in the α-carbon but not the ω-carbon atom (*viz.* α-methyl acetoacetate and β-ketoadipate).

Succinyl-*S*-pantetheine and succinyl-*S*-glutathione, even in high concentration, cannot replace succinyl-S-CoA as substrate for the transferase. Acetoacetyl-*S*-pantetheine is also inactive. Beinert and Stansly (19) have demonstrated that malonate, butyrate, β-hydroxybutyrate, and valerate, as well as succinate and acetoacetate, increase the formation of labeled acetoacetate from C^{14}-acetyl-S-CoA in the presence of crude heart fractions containing CoA transferase and thiolase. They interpret their results to indicate that these acids can replace succinate as substrate for CoA transferase. Since CoA transferase is strictly specific for the succinyl moiety, this interpretation is untenable, and other factors must have been operative.

Reaction with Acetoacetyl-S-CoA—The purified transferase catalyzes the transfer of CoA from acetoacetyl CoA to succinate (Curve A, Fig. 2) more rapidly than the reverse reaction. When tested under optimal conditions and without Mg^{++} ions (to avoid competitive chelation effects), the rate was 25 times faster with acetoacetyl CoA than with succinyl CoA as initial reactant (Table III). The reversibility of the reaction is demonstrated in the experiment of Curve B, Fig. 2.

Affinity of Substrates—These determinations were all performed in the absence of Mg^{++} because of the chelation of Mg^{++} by high concentrations of succinate and possibly acetoacetate. The K_m value, determined by the method of Lineweaver and Burk (20), was 1.6×10^{-5} M for acetoacetyl

[8] M. J. Coon, unpublished experiments.

CoA and 8.8×10^{-5} M for succinyl CoA. The concentrations required to saturate the transferase were 4.6×10^{-5} and 3.6×10^{-4} M, respectively. The affinity for acetoacetate and for succinate was much less. Accurate K_m values were not obtained for technical reasons. However, the rates given in parentheses, expressed as micromoles of CoA transferred per minute per mg. of enzyme, were observed for the concentration of free acid indicated as follows: succinyl CoA to acetoacetate, 1×10^{-3} M (0.18),

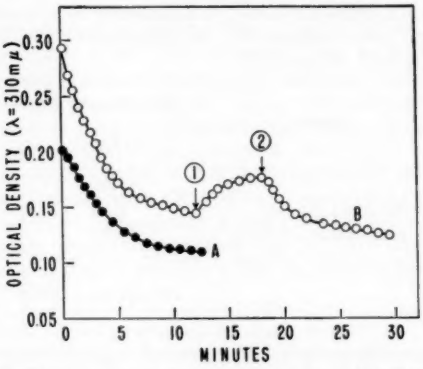

FIG. 2. Optical measurement of the reaction acetoacetyl-S-CoA + succinate ⇌ succinyl-S-CoA + acetoacetate. Silica cells (d = 0.5 cm.); temperature 25°. Curve A, the cells contained 100 μmoles of Tris-HCl buffer, pH 8.1, 0.07 μmole of acetoacetyl CoA (containing an additional 0.03 μmole of acetoacetyl-*S*-glutathione), 100 μmoles of potassium succinate, pH 8.0, 0.21 γ of CoA transferase (specific activity 572), and water to a final volume of 1.50 ml. The reaction was started at zero time by addition of enzyme. Curve B, the cells contained initially 100 μmoles of Tris-HCl buffer, pH 8.1, 0.20 μmole of acetoacetyl CoA (containing 0.08 μmole of acetoacetyl-*S*-glutathione), 0.75 μmole of potassium succinate, pH 8.0, 42 γ of transferase (specific activity 572), and water to a final volume of 1.50 ml. The reaction was started at zero time by addition of enzyme. At Arrow 1, 100 μmoles of potassium acetoacetate added; at Arrow 2, 10 μmoles of potassium succinate added. Thio ester was omitted from the reference cell in both experiments. Readings corrected for dilution after each addition.

5×10^{-3} M (0.36), 2×10^{-2} M (0.54), 5×10^{-2} M (1.30), 6.7×10^{-2} M (1.98); acetoacetyl CoA to succinate, 2.5×10^{-3} M (13.4), 2.5×10^{-2} M (21.5), 5×10^{-2} M (37.5), 6.7×10^{-2} M (48.6).

Effect of pH—As shown in Table IV, the reaction rate for the transfer of CoA from succinyl CoA to acetoacetate increased continuously over the range pH 7.0 to 9.1 as determined by the optical method. One factor contributing to this effect is the dissociation of a proton from acetoacetyl-S-CoA above pH 9.0, for, as shown by Stern (7), the pK′ of the latter is 9.45. At pH 7.5 and above, Mg^{++} appeared to inhibit the reaction. If,

as Loewus *et al.* (21) have demonstrated for oxalacetate and malic dehydrogenase, the keto form is the active substrate of the transferase, this effect may be more apparent than real and simply inherent in the optical

TABLE III

Relative Rates of Reactions Catalyzed by Transferase

In Experiment 1, the reaction mixture contained (in micromoles) Tris-HCl buffer, pH 8.1, 100; succinyl-S-CoA, 0.6; potassium acetoacetate, 100; and transferase, 4.2 γ (specific activity 572, originally 1000). 7.5 μmoles of $MgCl_2$ added as indicated. Final volume, 1.50 ml.; temperature, 23°. In Experiment 2, the conditions were the same as those for Curve A of Fig. 2.

Experiment No.	Substrate	CoA transferred per min. per mg. of enzyme	
		No Mg^{++}	5×10^{-3} M Mg^{++}
		μmoles	*μmoles*
1	Succinyl-S-CoA	1.98	1.44
2	Acetoacetyl-S-CoA	48.6	40.8 (51.1)*
Ratio, $\frac{\text{Experiment 2}}{\text{Experiment 1}}$		24.5	28.3 (35.5)

* The values in parentheses have been corrected (approximately) for chelation of added Mg^{++} by high concentrations of succinate, an effect which decreases the effective concentration of Mg^{++} and hence the extinction of acetoacetyl CoA.

TABLE IV

Effect of pH on Transferase Activity

Conditions as in Experiment 1, Table III. Tris-HCl buffer used throughout.

pH	CoA transferred per min. per mg. of enzyme	
	No Mg^{++}	5×10^{-3} M Mg^{++}
	μmoles	*μmoles*
7.0	0.48	0.56
7.5	1.00	0.80
8.1	1.98	1.44
9.1	3.05	1.71

method of assay in which the concentration of the enolate or chelate forms or both is measured (7).

Equilibrium Constants—The equilibrium constant of Reaction 1 is given by the expression

$$K = \frac{[\text{acetoacetyl-S-CoA}^-][\text{succinate}^=][H^+]}{[\text{succinyl-S-CoA}^-][\text{acetoacetate}^-]}$$

We have determined at different pH values, in the presence and absence of added Mg^{++}, the apparent equilibrium constant K', employing for the calculation the total concentration of reactants (ionized and unionized, chelated and unchelated) at equilibrium according to the equation

$$K' = \frac{(\text{acetoacetyl-S-CoA})(\text{succinate})}{(\text{succinyl-S-CoA})(\text{acetoacetate})}$$

TABLE V

Equilibrium Constant of Reaction Succinyl-CoA + Acetoacetate ⇌ Acetoacetyl-S-CoA + Succinate at pH 8.1 in Presence or Absence of Mg^{++}

The reaction mixture contained initially Tris-HCl buffer, pH 8.1 (0.10 M), $MgCl_2$ (5.3×10^{-3} M), potassium acetoacetate (66.7×10^{-3} M), succinyl-S-CoA (0.54×10^{-3} M in Experiments 1, 3, and 4; 0.41×10^{-3} M in Experiment 2), and potassium succinate (2.43×10^{-3} M in Experiments 1, 3, and 4; 2.11×10^{-3} M in Experiment 2). The reaction was started by addition of 60 γ of transferase (specific activity 880). Final volume, 1.5 ml.; temperature, 30°. pH (glass electrode) 8.10. Acetoacetyl-S-CoA formation measured at 310 mμ against a reference cell containing all components except succinyl-S-CoA. Equilibrium was attained in 7 to 10 minutes. A correction was made for 5 per cent loss of succinyl-S-CoA by spontaneous hydrolysis during this period. Silica cells, $d = 0.5$ cm.

Experiment No.	Equilibrium concentrations ($\times 10^{-3}$ M)				K' ($\times 10^{3}$)
	Acetoacetyl-S-CoA	Succinate	Acetoacetate	Succinyl-S-CoA	
1	0.095	2.55	66.5	0.42	8.67
2	0.075	2.44	66.6	0.31	8.84
3*	0.053	2.51	66.6	0.46	4.34
4†	0.098	2.55	66.5	0.42	8.96
Average (with Mg^{++})					8.82

* $MgCl_2$ omitted. It was not determined whether the other reactants were completely free of Mg^{++}.

† $MgCl_2$ (5.3×10^{-3} M) added to cells of Experiment 3 after equilibrium was reached.

At a given pH, K' was determined by measuring optically at $\lambda = 310$ mμ the formation of acetoacetyl-S-CoA from succinyl-S-CoA and acetoacetate with constant concentrations of CoA transferase. Since the initial concentration of all reactants was known, their equilibrium concentration could be calculated from the $+\Delta E_{310}$ by use of the molecular extinction coefficient (ϵ_{310}) of acetoacetyl-S-CoA determined under the same conditions. Typical results at pH 8.1 and 30° are shown in Table V. It is seen that the presence of Mg^{++}, which forms a chelate compound with acetoacetyl-S-CoA (4, 7), doubled the value of K', and that the same value

of K' was obtained whether Mg^{++} was present initially or was added after the reaction had attained equilibrium in its absence. The average value of K', in the presence of Mg^{++}, was 8.82×10^{-3}.

The effect of hydrogen ion concentration on the apparent equilibrium constant of Reaction 1 is shown in Table VI, both in the presence and absence of added Mg^{++}. From the equation $\log K' \simeq \log K + pH$, one might expect a plot of $\log K'$ *versus* pH to give a straight line of slope 1.0. Actually the experimentally determined values of Table VI when so plotted fall on a line of slope 0.24 (with Mg^{++}) and 0.21 (without Mg^{++}), indicating that the active concentrations of the reactants do not approximate the total concentrations within the narrow pH range investigated.

TABLE VI

Apparent Equilibrium Constant K' As Function of pH in Presence or Absence of Mg^{++} at 30°

The experimental conditions were the same as those of Table III except that the initial concentrations of succinyl-S-CoA and succinate, respectively, were as follows: pH 7.0 (0.72 and 1.24×10^{-3} M), pH 7.5 (0.54 and 1.10×10^{-3} M), pH 9.20 (0.09 and 2.02×10^{-3} M). Also, 0.10 M glycine buffer was used at pH 9.20.

pH	K' ($\times 10^3$)	
	Without Mg^{++}	With Mg^{++}
7.0	2.8	4.9
7.5	4.2	5.9
8.1	4.3	8.8
9.2	8.6	15.0

Distribution—CoA transferase is also present in kidney as demonstrated by our observation (18) that acetoacetate synthesis by pig kidney extract from acetyl-S-CoA (generated from acetyl phosphate and catalytic amounts of CoA-SH with transacetylase) is dependent on the presence of succinate and succinyl CoA deacylase. Moreover, although these kidney extracts form citrate from acetoacetate, ATP, and CoA, in the presence of oxalacetate, the yield of citrate is considerably augmented by addition of succinate, since the P enzyme system (22, 23) is also present and generates succinyl CoA. Experiments so far have failed to demonstrate CoA transferase in pigeon breast muscle, rabbit skeletal muscle, or pigeon brain. They have established that pigeon brain extract possesses the acetoacetate-activating enzyme (18) which synthesizes acetoacetyl-S-CoA from acetoacetate, CoA, and ATP.

Activators and Inhibitors—No activators of CoA transferase are known. The enzyme is not inhibited by 10^{-3} M potassium EDTA, confirming that Mg^{++} is not required for activity. It is not inhibited by 10^{-2} M iodoacetate.

Attempts to apply the direct optical assay to crude extracts by inhibiting thiolase with iodoacetate (6) have given variable results.

DISCUSSION

The free energy (ΔF°) of Reaction 1 at a given pH can be calculated from the equation $\Delta F^\circ = -RT \ln K'$. At pH 7.0 and 25° the free energy change of Reaction 1 is, therefore, $\Delta F^\circ = -1360 \log (2.83 \times 10^{-3}) = +3470$ calories. Within the range pH 7.0 to 9.2 the average decrease in the free energy change of Reaction 1 per unit increase in pH was 300 calories. The discrepancy between this and the theoretical value of 1380 calories is due to changes in the degree of ionization of the reactants. The free energy of hydrolysis of acetoacetyl-S-CoA is obviously greater than that of succinyl-S-CoA. Assuming that the free energy of hydrolysis of succinyl-S-CoA is about equal to that of acetyl-S-CoA,[9] for which a revised value of −8200 calories (pH 7.0 and 25°) has been calculated by Burton (25) from equilibrium data, the free energy of hydrolysis of acetoacetyl-S-CoA would be −11,670 calories. It is relevant that, by coupling CoA transferase with the P enzyme system (22, 23), this energy can be transferred to the pyrophosphate bond of ATP, 1 mole of which may be synthesized for each mole of acetoacetyl-S-CoA generated during fatty acid oxidation.

There can be little doubt that CoA transferase plays an active rôle in the utilization of acetoacetate by peripheral tissues (particularly heart and kidney), by converting it to acetoacetyl-S-CoA which may be either reduced to fatty acids via the fatty acid cycle or oxidized via the citric acid cycle. The latter in turn generates the necessary succinyl-S-CoA by oxidation of α-ketoglutarate. The utilization of acetoacetate (ketone bodies) by the heart has been demonstrated *in situ* both in man (26) and dog (27, 28). Bing *et al.* (26) have determined that the rate of blood ketone utilization by the human heart is normally 0.50 to 6.1 μmoles per 100 gm. of heart per minute at 37°. Assuming (*a*) that 15 per cent of the heart is protein, (*b*) that 0.09 per cent of the protein is transferase, and (*c*) a turnover number of 450 (calculated from data in this paper for pH 7.5 and assuming a 3-fold increase at 37°), then 60 μmoles of acetoacetate could be utilized per minute by 100 gm. of heart if the blood level were 10^{-3} M. By extrapolation, this is compatible with the above values determined at blood levels of 3 to 10×10^{-5} M.

Materials

Pabst CoA which assayed 50 to 65 per cent pure and contained up to 20 per cent (molar basis) glutathione was used. Succinyl-S-CoA, maleyl-S-

[9] This is indicated by the close agreement between the K' values for the P enzyme reaction (22) and the aceto-CoA-kinase reaction (24).

CoA, and crotonyl-S-CoA were prepared by reaction of the anhydride with CoA-SH according to the procedure of Simon and Shemin (29). In the case of succinyl CoA, the actual succinyl-S-CoA content of the solution was about 50 per cent of the total thio ester formation calculated from the disappearance of sulfhydryl. Since succinyl CoA is relatively unstable, the solution was kept frozen at neutral pH and used within 2 weeks. Acetoacetyl-S-CoA was prepared by reacting diketene with CoA-SH at pH 5.0 to 6.0 (*cf.* (6)). It was stored at −20°. We are indebted to Dr. J. N. Eisendrath, Aldrich Chemical Company, Milwaukee, for a generous sample of purified diketene. Partly purified D-amino acid apooxidase from pig kidney (13) was kindly supplied by Dr. I. Zelitch.

SUMMARY

1. The preparation from pig heart and properties of highly purified CoA transferase, the enzyme which catalyzes reversible CoA transfer between succinate and β-keto acids, are described.

2. The enzyme is strictly specific for succinyl-S-CoA and the C_4 to C_6 β-ketoacyl-S-CoA compounds.

3. The equilibrium constant of the CoA transferase reaction, which is markedly in favor of succinyl-S-CoA and acetoacetate, has been determined and the free energy change has been calculated. The free energy of hydrolysis of the thio ester bond of acetoacetyl-S-CoA is greater than that of succinyl-S-CoA by some 3500 calories.

4. The distribution of CoA transferase and its rôle in the utilization of blood acetoacetate by heart are discussed.

The authors wish to thank Professor S. Ochoa for his helpful interest.

Addendum—Since submission of the manuscript, CoA transferase has been shown to be present in extracts of dog skeletal muscle and ox adrenal gland, tissues known to utilize blood ketones. These extracts catalyze the conversion of acetoacetyl-S-CoA to succinyl-S-CoA as measured by the modified direct optical assay. (J. R. Stern, unpublished experiments.)

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XUM

OPTICAL PROPERTIES OF ACETOACETYL-*S*-COENZYME A AND ITS METAL CHELATES*

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(Received for publication, November 7, 1955)

Acetoacetic thio esters of CoA[1] (1–3) and possibly pantetheine (4, 5) play an important rôle in fatty acid metabolism. Effective use has been made of the optical properties of these thio esters in developing sensitive assays (6) for the enzymes concerned in their metabolism.

Lynen and collaborators (7) were the first to discover that acetoacetic thio esters, specifically synthetic *S*-acetoacetyl-*N*-acetyl-β-mercaptoethyl-

(1) $CH_3—\overset{\overset{O}{\|}}{C}—CH_2—\overset{\overset{O}{\|}}{C}—SR \rightleftharpoons CH_3—\overset{\overset{OH}{|}}{C}=CH—\overset{\overset{O}{\|}}{C}—SR \rightleftharpoons$

$$CH_3—\overset{\overset{O^-}{|}}{C}=CH—\overset{\overset{O}{\|}}{C}—SR + H^+$$

amine, possess a strong absorption band between 280 and 320 mμ, with a maximum around 303 mμ. Since the intensity of this band was dependent on pH, increasing with increasing pH, they attributed it to formation of the enolate ion, as indicated by reaction (1). The pK′ of *S*-acetoacetyl-*N*-acetyl-β-mercaptoethylamine was determined to be 8.5. Acetoacetyl-S-CoA prepared enzymatically (8, 9) or synthetically (1) was found to have a similar absorption band. Stern *et al.* (10) showed that the intensity of this band was greatly increased in the presence of Mg^{++}. These observations have been confirmed by Beinert (9) and extended to include the C_6 and C_8 β-ketoacyl-S-CoA compounds.

This paper deals with the optical properties of metal chelates[2] of aceto-

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[1] The following abbreviations are used: CoA (or CoA-SH), coenzyme A (reduced); R-SH, thiol compound; AcAc, acetoacetate; *M*, metal; ε, molecular extinction coefficient; *E*, optical density; λ_{max}, wave-length at which ε is maximum.

[2] The term chelate is used in its strict chemical sense (*cf.* (11)). Although the compounds described have not been isolated, their behavior in solution is completely in accord with that of known metal chelates of β-diketones (11–13).

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acetyl-S-CoA and related thio esters. The significance of these properties in the interpretation of optical assays will be discussed.

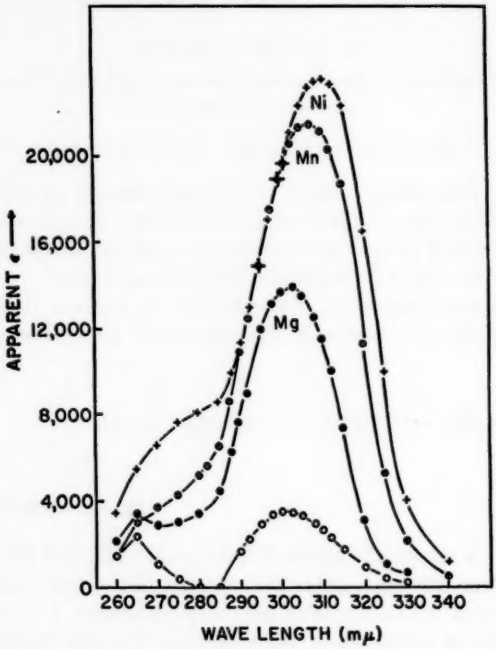


FIG. 1. Effect of divalent ions on the absorption spectrum of acetoacetyl-S-CoA. These are difference absorption spectra obtained by first measuring the enolate (or enolate + chelate) spectrum at pH 8.1, then adding HCl (final concentration, 0.1 N) to obtain the non-enolate absorption spectrum. The latter is subtracted from the former. The difference spectrum between 260 and 280 mμ may be attributed in part to changes in absorption of the adenosine moiety of the thio ester. The lowest curve represents the acetoacetyl-S-CoA spectrum in absence of metal. Metal indicated added as chloride salt, final concentration 5×10^{-3} M. Acetoacetyl-S-CoA concentration, 0.68×10^{-4} M (total acetoacetic thio ester concentration, 0.76×10^{-4} M). Standard optical conditions.

EXPERIMENTAL

Absorption Spectrum—Fig. 1 shows the difference absorption spectrum at pH 8.1 of acetoacetyl-S-CoA in the range 260 to 340 mμ and the effect of various divalent metal ions (concentration, 5×10^{-3} M) on it. It is evident that Mg^{++}, Mn^{++}, and Ni^{++}, in order of increasing effectiveness, cause a great increase in the intensity of the enolate ion absorption as well as some shift in λ_{max}. This increase in absorption may be attributed to

the formation of a 1:1 metal chelate[3] (reaction (2)) which possesses a greater degree of conjugation than the chelating agent, *i.e.* acetoacetyl-S-CoA.

(2) $$M^{++} + \text{acetoacetyl-S-CoA}^{-} \rightleftharpoons M\text{-acetoacetyl-S-CoA}^{+}$$

The λ_{max} of acetoacetyl-S-CoA and of its Mg chelate both occurred in the range 300 to 303 mμ. For the Co, Mn, and Ni chelates of acetoacetyl-S-CoA, λ_{max} was 306 ± 2 mμ, 308 mμ, and 309 to 310 mμ, respectively.

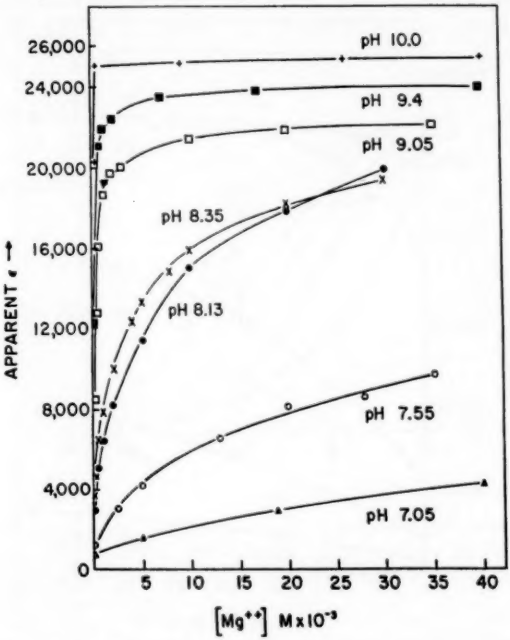


FIG. 2. Effect of Mg^{++} concentration and pH on the extinction of acetoacetyl-S-CoA solutions at wave-length 310 mμ.

This small but definite shift of λ_{max} toward the red indicates that the Co, Mn, and Ni chelates may differ in structure from the Mg chelate.

[3] In all experiments described the concentration of metal was high compared to that of chelating agent, so negligible quantities of $M(AcAcCoA)_2$ were present (*cf.* [4]). For example, consider the further reaction $M AcAcCoA^{+} + AcAcCoA^{-} \rightleftharpoons M(AcAcCoA)_2$ where $(M^{++})_e^1 \simeq 5 \times 10^{-3}$ M and $(AcAcCoA^{-}) \simeq 10^{-5}$ M. Even if $K_e^2 = (M(AcAcCoA)_2)/(MAcAcCoA^{+})(AcAcCoA^{-}))$ were approximately equal to K_e' (= 2750), the ratio $(M(AcAcCoA)_2)/(MAcAcCoA^{-})$ would not exceed $2750 \times 10^{-5} \simeq 0.028$. Further evidence that a 1:1 chelate is formed under the experimental conditions is given in the text.

These changes in absorption spectra resemble those described for metal chelates of a simpler β-diketone, acetylacetone (12, 13), which possesses an ultraviolet absorption band whose λ_{max} = 273 mμ in ethanol. However, in the case of the Co and Ni chelate compounds of acetylacetone there is a more pronounced shift of λ_{max} to the red.

Effect of Mg^{++} Concentration on Extinction—The effect of Mg^{++} concentration on the extinction of acetoacetyl-S-CoA and acetoacetyl-S-

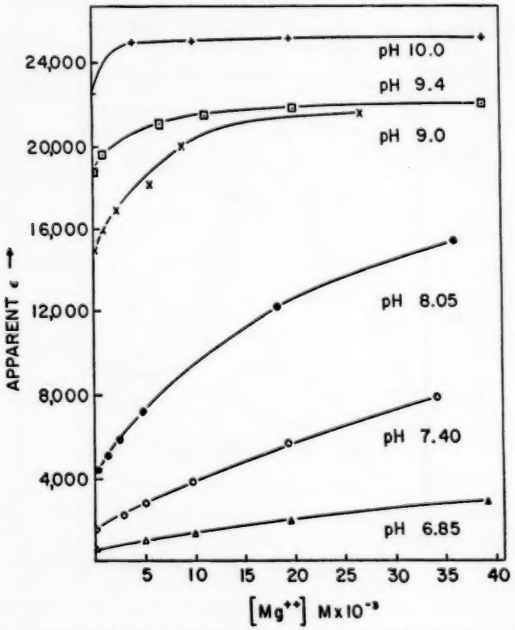


FIG. 3. Effect of Mg^{++} concentration and pH on the extinction of acetoacetyl-S-pantetheine solutions at wave-length 310 mμ.

pantetheine at λ = 310 mμ is shown in Figs. 2 and 3 for a number of pH values. It is seen that, at any given pH, the concentration of chelate increases as the Mg^{++} concentration increases and that for a given concentration of Mg^{++} the extinction is greater the more alkaline the pH, as is the case in the absence of Mg^{++}.

Molecular Extinction Coefficient—The molecular extinction coefficient (ε) of the Mg-acetoacetyl-S-CoA chelate was determined by measuring its extinction under conditions for which chelate formation may be considered to be complete, *i.e.* at very alkaline pH or at less alkaline pH in the pres-

ence of a large excess of Mg^{++}. By this method (*cf.* Figs. 2, 3, and 4) the true ϵ_{310} for the Mg chelates of both acetoacetyl-S-CoA and acetoacetyl-*S*-pantetheine was found to be 25,000. It is noteworthy that the ϵ_{310} obtained by this method for the enolate ion form of the two thio esters is also 25,000. At the λ_{max} indicated the values of ϵ were as follows: $\epsilon_{300} \simeq$ 32,000 for acetoacetyl-S-CoA and $\epsilon_{303} \simeq 30{,}000$ for its Mg chelate.

Effect of pH—The effect of pH on the apparent molecular extinction coefficient, ϵ',[4] of acetoacetyl-S-CoA and acetoacetyl-*S*-pantetheine in the absence and presence of various Mg^{++} concentrations is presented in Fig.

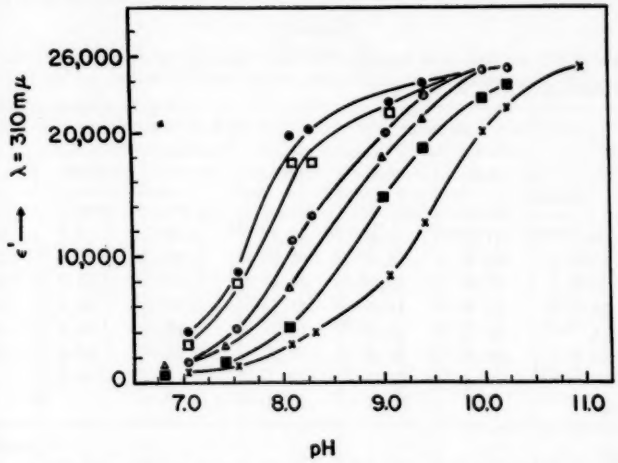


FIG. 4. The effect of pH on the extinction of acetoacetic thio esters. Acetoacetyl-S-CoA, ×, no metal; ⊙, 5.0×10^{-3} M $MgCl_2$; □, 20×10^{-3} M $MgCl_2$; ●, 35×10^{-3} M $MgCl_2$. Acetoacetyl-*S*-pantetheine, ■, no metal; △, 5×10^{-3} M $MgCl_2$. ◑, points common to upper four curves.

4. In each instance ϵ' increased continuously with pH. The pH at which ϵ' has half the maximal value corresponds to the pK′ values of the acetoacetic thio esters or their metal chelates. The pK′ of acetoacetyl-S-CoA is therefore 9.45. The value of pK′ decreases with increasing Mg^{++} concentration, a factor of biological significance. Since Lynen (1) had reported a pK′ of 8.50 for *S*-acetoacetyl-*N*-acetyl-β-mercaptoethylamine, the problem arose whether this difference of pK′ values was the result of metal contamination or a property of the thiol moiety of the acetoacetic thio ester. The effect of pH on the ϵ' of acetoacetyl-*S*-pantetheine (4) was therefore examined (Fig. 4). At any given pH the ϵ' of acetoacetyl-

[4] ϵ' is defined as $\log I_0/I = \epsilon' cd$ where c is the molar concentration of total acetoacetic thio ester.

S-pantetheine was greater than that of acetoacetyl-S-CoA and the effect of a given Mg^{++} concentration on ϵ' was less, since the pK′ of acetoacetyl-*S*-pantetheine, 8.85, was less than that of acetoacetyl-S-CoA and also greater than that of *S*-acetoacetyl-*N*-acetyl-β-mercaptoethylamine. These results emphasize the influence of groups in the thiol moiety on the pK′ of acetoacetic thio esters.

Calculation of Stability Constant—Let A, A^-, and AM^+ represent the keto, enol, and chelate forms, respectively, of acetoacetyl-S-CoA and A_T the sum of the three forms. At any given pH, the dissociation constant

TABLE I

Effect of pH on Degree of Enolization and Chelation of Acetoacetyl-S-CoA

Conditions as in Fig. 2.

pH	No metal*		5×10^{-3} M Mg^{++}			K_c
	Keto	Enol†	Keto	Enol	Chelate	
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	
7.05	97.6	2.4	93.9	0.37	5.7	3080
7.55	95.1	4.9	83.3	1.05	15.6	2980
8.13	87.8	12.2	54.4	2.40	43.2	3460
8.35	84.4	15.6	47.1	3.34	49.6	2970
9.15	66.2	33.8	19.5	9.83	70.7	1450
9.40	50.4	49.6	7.51	6.70	85.8	2560
10.00	20.0	80.0	~0	~0	~100.0	
10.25	12.7	87.3				
Average.....						2750

* E not affected by addition of potassium versenate.

† Calculated from the equation per cent enol = $100\epsilon'/25{,}000$.

(K_H) of the reaction $A \rightleftharpoons A^- + H^+$ is given by the equation

$$K_H = \frac{(A^-)(H^+)}{(A)} \tag{3}$$

while at any given concentration of metal (M^{++}), the stability constant (K_c) of the reaction $A^- + M^{++} \rightleftharpoons AM^+$ is given by the equation

$$K_c = \frac{(AM^+)}{(A^-)(M^{++})} \tag{4}$$

Also,

$$(A_T) = (A) + (A^-) + (AM^+) \tag{5}$$

Under the experimental conditions $(M_T) \simeq (M^{++}) > (A)$ or (A^-). Since at λ_{310} $\epsilon_{AM^+} = \epsilon_{A^-} = 25{,}000$, the sum $((AM^+) + (A^-))$ can be determined

by measuring the extinction (at constant pH and (M^{++})), applying Beer's law ($\log I_0/I = \epsilon cd$), and solving for c. Also, (A_T) can be measured enzymatically and (A) calculated from equation (6) as follows:

$$(A) = (A_T) - ((AM^+) + (A^-)) \tag{6}$$

(A^-) can now be determined from equation (3), since pK′, pH, and (A) are known. This then permits (AM^+) to be computed, since $((AM^+) + (A^-))$ is known. Finally K_c can be computed.

Table I gives (*a*) the proportion of keto and enol forms of acetoacetyl-S-CoA as a function of pH in the absence of metal and (*b*) the proportions

TABLE II

Effect of pH on Degree of Enolization and Chelation of Acetoacetyl-S-pantetheine

Conditions as in Fig. 3.

pH	No metal*		5×10^{-3} M Mg^{++}			K_c
	Keto	Enol†	Keto	Enol	Chelate	
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	
6.85	98.0	2.0	96.2	0.86	2.91	673
7.40	94.1	5.9	88.8	3.16	8.03	511
8.05	82.3	17.7	70.7	11.2	18.1	345
9.00	40.8	59.2	27.1	38.2	34.7	193
9.40	24.8	75.2	8.2	29.0	62.8	432
10.00	10.0	90.0	~0	~0	~100.0	
10.25	6.0	94.0				
Average.....						431

* *E* not affected by addition of potassium versenate.

† Calculated from the equation per cent enol = $100\epsilon'/25{,}000$.

of the keto, enol, and chelate forms in the presence of 5×10^{-3} M Mg^{++}, computed as indicated above. It is seen that the values of K_c are reasonably constant and independent of pH. Table II presents analogous data for acetoacetyl-*S*-pantetheine. Here the values of K_c show greater spread.

The standard free energy (ΔF°) of the formation of the 1:1 Mg chelate can be calculated from the equation $\Delta F^\circ = -RT \ln K_c$. For Mg-acetoacetyl-S-CoA $\Delta F^\circ = -4670$ calories per mole, while for Mg-acetoacetyl-*S*-pantetheine $\Delta F^\circ \simeq -3580$ calories per mole.

Stability of Metal Chelates—At alkaline pH acetoacetic thio esters are unstable and slowly hydrolyze according to reaction (7).

$$\text{Acetoacetyl-SR}^- + \text{H}_2\text{O} \rightarrow \text{acetoacetate}^- + \text{HSR} \tag{7}$$

The time-course of spontaneous hydrolysis of acetoacetyl-S-CoA at pH

8.1 is illustrated in Fig. 5. The metal chelates of acetoacetyl-S-CoA also hydrolyze spontaneously, at varying rates, at alkaline pH according to reaction (8).

(8) M-acetoacetyl-$SR^+ + H_2O \rightarrow M^{++} +$ acetoacetate$^-$ + HSR

The stability of various metal chelates at pH 8.1 and 25° is shown in Fig. 5. The Ni chelate was the most stable, followed by the Co, Mn, and Mg

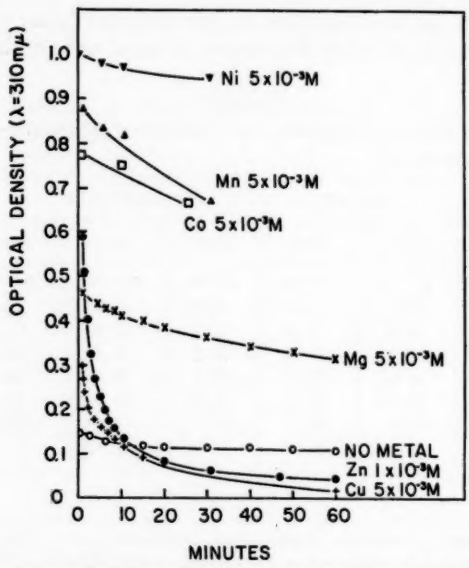


FIG. 5. Time-course of spontaneous hydrolysis of acetoacetyl-S-CoA and various metal chelates. The thio ester was added at zero time to solutions containing the indicated concentration of metal in 0.067 M Tris-HCl buffer, pH 8.1. Final acetoacetyl-S-CoA concentration, 0.68×10^{-4} M (total acetoacetic thio ester 0.76×10^{-4} M). Volume 1.50 ml.; temperature 25°.

chelates which possess similar stability. The Cu and, particularly, the Zn chelates were much more unstable and it was not practical to determine their difference spectra or λ_{max} in the Beckman DU spectrophotometer.

Reaction of Hg^{++} with Acetoacetyl-S-CoA—It was found that addition of Hg^{++} in concentrations higher than 10^{-6} M to solutions of acetoacetyl-S-CoA (1.6×10^{-4} M) or to solutions containing its Mg chelate (0.8×10^{-4} M total thio ester) at pH 8.1 resulted instantaneously in a partial decrease of the extinction of these compounds. 1 to 2×10^{-4} M $HgCl_2$ completely abolished the enolate ion absorption band of acetoacetyl-S-CoA

or its Mg chelate. The decrease in extinction at 310 mμ was proportional to the amount of Hg^{++} added and it was determined that, to achieve complete "titration" of the E_{310} of these compounds, just more than 1 mole of Hg^{++} was required per mole of acetoacetyl-S-CoA. This observation suggested that the Hg chelate of acetoacetyl-S-CoA was extremely unstable and hydrolyzed immediately. This interpretation was supported (*a*) by the disappearance of the typical enolate ion (or chelate) absorption band and (*b*) by the fact that, after "titration" of the E_{310} with Hg^{++} and addition of potassium versenate to bind any excess Hg^{++}, enzymatic assay for acetoacetyl-S-CoA with L(+)-β-hydroxybutyryl-S-CoA dehydrogenase was negative. Since Hg^{++} also hydrolyzed solutions of the Mg chelate, it must have a greater affinity for acetoacetyl-S-CoA than Mg^{++}; *i.e.*, the stability constant of the Hg chelate (reaction (9)) is greater than that of the Mg chelate. The fact that Hg^{++} does not act catalytically in hydrolyzing acetoacetyl-S-CoA and other acetoacetic thio esters can be explained by the binding of Hg^{++} by the liberated thiol according to the following reaction sequence:

(9) $$Hg^{++} + \text{acetoacetyl-}SR^- \rightleftharpoons \text{Hg-acetoacetyl-}SR^+$$

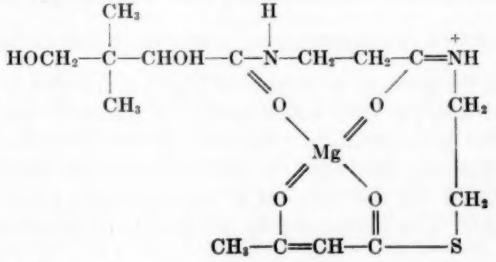
(10) $$\text{Hg-acetoacetyl-}SR^+ + H_2O \rightarrow Hg^{++} + \text{acetoacetate}^- + HSR$$

(11) $$Hg^{++} + HSR + 2Cl^- \rightleftharpoons HgSR^+ + HCl + Cl^-$$

Hg^{++} also hydrolyzed the Ni, Mn, and Co chelates of acetoacetyl-S-CoA.

DISCUSSION

The experiments presented above permit the conclusion that a 1:1 chelate is formed between acetoacetic thio esters of CoA or pantetheine and certain divalent ions. Possibly an "internal" chelate is formed in which the ligand groups are the enol and carbonyl oxygen atoms of the acyl moiety and the carbonyl oxygen atoms (or possibly nitrogen atoms) of the two peptide bonds, as follows:



This structure of the chelate would differ drastically from that of the enol

form and would not be possible with *S*-acetoacetyl-*N*-acetyl-β-mercaptoethylamine.

Optical tests of enzymatic reactions of acetoacetyl-S-CoA or acetoacetyl-*S*-pantetheine measure the change in concentration of the enol form or (if a particular divalent metal ion is present) of the sum of the enol and chelate forms. The possibility exists that the keto form may be the actual substrate, in which case one is measuring not the enzymatic reaction but the resultant and instantaneous chemical change in the equilibrium concentration of the enol (± chelate) form. Since none of the enzymes concerned, dehydrogenase, thiolase, and CoA transferase (acetoacetyl-succinic thiophorase[5]), requires Mg^{++} or other divalent ion, the chelate is not an obligatory substrate. Although the problem of distinguishing between keto and enol substrates invites solution by isotope experiments, some experiments already reported (15) with CoA transferase are of interest in this regard. The apparent equilibrium constant (K') of reaction (12) was found to increase continuously over the range pH 7.0 to 9.2 when

TABLE III

Apparent Equilibrium Constant K′ of CoA Transferase Reaction Succinyl-S-CoA + Acetoacetate ⇌ Acetoacetyl-S-CoA + Succinate

Recalculated from data of Stern *et al.* (15), assuming that only the keto form of acetoacetyl-S-CoA is a substrate of CoA transferase. Concentration of keto form calculated by method given in the text.

pH	$K' \times 10^8$	
	No metal	5×10^{-3} M Mg^{++}
7.00	2.8	4.6
7.50	4.0	4.9
8.10	3.9	4.8
9.20	5.5	3.0

(12) Succinyl-S-CoA$^-$ + acetoacetate$^-$ ⇌ acetoacetyl-S-CoA$^-$ + succinate$^=$ + H^+

determined in the presence or absence of Mg^{++}, and a plot of log K' *versus* pH suggested that the total concentration of reactants did not approximate the active concentrations. However, if one recalculates K' on the assumption that only the keto form participates in the equilibrium, then, as shown in Table III, the values of K' are reasonably constant and independent of pH. This indicates that the keto form of acetoacetyl-S-CoA

[5] This name has been proposed by a Committee on Nomenclature, 2nd International Conference on Biochemical Problems of Lipides, Ghent, Belgium, July, 1955.

may be the substrate of CoA transferase. Consistent with this hypothesis was the further observation that the inhibition by Mg^{++} of the rate of CoA transferase activity increased with pH and paralleled the suppression of the keto concentration through increasing chelation (Table I). However, this effect could also be explained by assuming that the chelate, though not a substrate, had some affinity for the enzyme.

These experiments emphasize the importance of the metallic environment in measuring by optical or other means transformations of acetoacetic thio esters. It is essential to eliminate Hg^{++}, Zn^{++}, and Cu^{++} ions from the reaction. The Ni, Mn, and Mg chelates, being the most stable, are best suited to increasing the sensitivity of optical measurements. The rapid hydrolysis of acetoacetic thio esters and their chelates by Hg^{++} suggests that this reaction may play a significant rôle in some toxic and inhibitory cellular actions of Hg^{++} previously explained solely on the basis of mercaptide formation with essential thiol groups of coenzymes or enzymes.

Methods

Acetoacetyl-S-CoA was made by reacting diketene with CoA (Pabst) at pH 6.0 (1). The resultant thio ester solution contained at least 80 per cent acetoacetyl-S-CoA and not more than 10 per cent acetoacetyl-*S*-glutathione. The total acetoacetic thio ester content was calculated from its extinction at $\lambda = 310$ mμ in 0.067 M Tris-HCl buffer, pH 8.1 and 5×10^{-3} M $MgCl_2$, with $\epsilon_{310} = 11{,}400$ (*cf.* Fig. 1). Acetoacetyl-S-CoA was determined quantitatively at pH 6.6 by measuring DPNH oxidation in the presence of partially purified heart L(+)-β-hydroxybutyryl-S-CoA dehydrogenase (10) (β-hydroxybutyryl dehydrogenase[5]). Acetoacetyl-*S*-pantetheine was made by reacting diketene and pantetheine. Pantethine was kindly supplied by Dr. O. D. Bird of Parke, Davis and Company, Detroit. It was essentially pure by analysis and had a microbiological potency of 23,800 LBF units per mg. Pantethine solutions were reduced as follows: For each ml. of 0.02 M pantethine 4 mg. of sodium borohydride were added and the solution was kept for 40 minutes at room temperature, when maximal reduction occurred. The yield of pantetheine was 65 per cent of theoretical. The solution was acidified to destroy excess borohydride, then brought to pH 6.0 with M potassium bicarbonate. Acetoacetyl-*S*-pantetheine was determined enzymatically with heart dehydrogenase (4, 5). Acetoacetic thio esters are quite stable at acid pH. Their solutions can be stored at pH 6 or less and $-15°$ for several months. All measurements were made at 25° in a Beckman DU spectrophotometer with wave-length calibration, silica cells of light path 0.5 cm. and a total volume of 1.50 ml. of solution being employed.

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SUMMARY

1. The effect of H^+ ion concentration and of metals on the absorption spectrum, extinction, and stability of acetoacetyl-S-CoA and acetoacetyl-*S*-pantetheine is described.

2. Acetoacetic thio esters are chelating agents and form metal chelates with Mg^{++}, Mn^{++}, Co^{++}, Ni^{++}, Cu^{++}, Zn^{++}, and Hg^{++}.

3. The dissociation constants of acetoacetyl-S-CoA and acetoacetyl-*S*-pantetheine and the stability constants of their Mg chelates have been determined by optical methods. The molecular extinction coefficients of the enol and Mg chelate forms have also been determined.

4. The hydrolysis of acetoacetyl-S-CoA and its metal chelates by Hg^{++} is described and its mechanism is discussed.

5. The significance of these optical properties in the interpretation of optical methods of enzyme assay is discussed.

The author is grateful to Dr. I. M. Klotz for valuable suggestions concerning the section on calculations.

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THE EFFECT OF GUANOSINE DIPHOSPHATE AND TRIPHOSPHATE ON THE INCORPORATION OF LABELED AMINO ACIDS INTO PROTEINS*

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We have previously described an enzyme system from rat liver which catalyzes the incorporation of labeled amino acids into proteins under anaerobic conditions (2, 3). This system consists of two liver cell fractions, the soluble cell fraction and the microsomes, to which are added a labeled amino acid, adenosine triphosphate,[1] and an ATP-generating system, such as phosphocreatine and creatine kinase. The microsome fraction contains ribonucleoprotein particles which appear to be the site of formation of the labeled proteins (4). The soluble cell fraction contains enzymes which catalyze the carboxyl activation of specific amino acids by ATP (5, 6).

Before attempting to separate the enzymes of the soluble cell fraction which are essential for the formation of labeled proteins, it was important to investigate the cofactor requirements of the system further. By a purification of the two liver cell fractions, it has now been found that guanosine di- or triphosphate is required in addition to ATP.

The requirement for ATP had been demonstrated in a system containing microsomes and a dialyzed soluble cell fraction (2). The addition of ATP to this partially dialyzed system restored the incorporation to the level in the crude system, but the possibility remained that the partially dialyzed system contained adequate amounts of other cofactors essential for amino acid incorporation. Of the various known cofactors which were added along with ATP, guanosine diphosphate was found to give a small increase in the labeling of the proteins. Our interest in testing GDP arose from the finding of Sanadi *et al.* (7) that it is the cofactor in the phosphorylation of ADP coupled to the breakdown of succinyl coenzyme A. To determine

* Supported by grants-in-aid from the American Cancer Society and the Atomic Energy Commission. This is publication No. 867 of the Cancer Commission of Harvard University. A preliminary report of this investigation has been published (1).

[1] The following abbreviations have been used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine-5′-phosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; GMP, guanosine-5′-phosphate; PEP, phosphoenolpyruvate.

whether GDP was indeed a cofactor in the formation of labeled proteins in the liver system, it was necessary to find a way to prepare microsomes and soluble cell proteins more nearly free of low molecular weight substances, including GDP. This was accomplished by sedimentation of the microsomes from a dilute extract and precipitation of the active proteins in the soluble cell fraction at pH 5.2. The mixture of proteins which precipitate at this pH from the soluble cell fraction will be referred to as the "pH 5 enzymes."

When the pH 5 enzymes were combined with microsomes from a dilute extract plus ATP and an ATP-generating system, there was only a slight incorporation of the labeled amino acid into proteins, but the addition of GDP or guanosine triphosphate restored the activity to the level in the crude system.

This paper describes the method of preparation and some characteristics, especially the nucleotide requirement, of the pH 5 enzymes-microsome system for the formation of labeled proteins.

Materials and Methods

Materials—L-Leucine-1-C^{14}, L-isoleucine-1-C^{14}, L-valine-1-C^{14}, glycine-1-C^{14}, DL-alanine-1-C^{14}, and DL-phenylalanine-1-C^{14} were prepared by Dr. Robert B. Loftfield (8, 9) from $BaC^{14}O_3$ obtained from the Oak Ridge National Laboratory on allocation from the Atomic Energy Commission. The L-leucine-C^{14} had a specific activity of approximately 3 mc. per mmole, and the others ranged from 1 to 4.5 mc. per mmole.

We are very much indebted to Dr. D. R. Sanadi for providing GDP and GTP before these became commercially available. Dr. Sanadi also contributed the GDP phosphorylation enzyme and an acetone powder of beef heart mitochondria for the preparation of nucleoside diphosphate kinase by a method which he kindly described for us in advance of publication (10).

Pyruvate kinase was kindly supplied by Dr. J. D. Gregory, and a *Streptococcus faecalis* R extract containing carbamate kinase by Dr. M. E. Jones (11). Creatine kinase was prepared by the method of Kuby *et al.* (12). All enzymes were dialyzed free of ammonium ions before use.

We are indebted to Dr. E. Cabib and Dr. L. F. Leloir for a gift of guanosine diphosphate mannose (13), to Dr. J. D. Gregory for inosine diphosphate and xanthosine diphosphate, and to Dr. W. E. Cohn and Dr. D. M. Brown for samples of guanosine-5′-phosphate. GDP, GTP, crystalline disodium ATP, and inosine-5′-phosphate and triphosphate were obtained from the Sigma Chemical Company, guanosine-3′-phosphate from the Schwarz Laboratories, and cytidine and uridine nucleotides from the Pabst Laboratories, partly as gifts from Dr. S. A. Morell. Nucleotides which were active in incorporation were analyzed by determining the absorption

spectrum in the region 220 to 320 mμ with a Cary recording spectrophotometer. Paper electrophoresis at pH 3.8 showed the presence of a small amount of GMP in the Sigma GDP, and of GDP in the Sigma GTP preparations used. All solutions of nucleoside di- and triphosphates were made up with 1 mole of $MgCl_2$ added per mole of nucleotide, so that addition of these solutions would not lower the magnesium ion concentration of the incubation mixture. All the solutions used in the incubation mixture were adjusted to approximately pH 7.4, by using KOH to neutralize the acids.

Carbamyl phosphate, dilithium salt, was the gift of Dr. L. Spector (11). Succinyl coenzyme A was prepared by the method of Simon and Shemin (14). Phosphocreatine, sodium salt, was obtained from the Sigma Chemical Company, and phosphoenolpyruvate, from the California Foundation for Biochemical Research, as the silver barium salt which was converted to the potassium salt.

Preparation of Microsomes and pH 5 Enzymes—Approximately 18 gm. of rat liver were homogenized, 3.5 gm. at a time, with 2.3 volumes of buffered medium under conditions described previously (2), except that the medium contained 0.35 M sucrose, 0.035 M $KHCO_3$, 0.004 M $MgCl_2$, and 0.025 M KCl (Medium A). The homogenate was centrifuged at 15,000 × *g* for 10 minutes at 0°. The supernatant fluid containing the microsomes and the soluble cell fraction was removed and diluted with 3 volumes of an unbuffered medium containing 0.90 M sucrose, 0.004 M $MgCl_2$, and 0.025 M KCl (Medium B). The diluted extract was centrifuged at 105,000 × *g* ($R_{average}$) for 1 hour at 0–3° in a Spinco preparative ultracentrifuge. The clear supernatant fluid containing the soluble cell fraction was pipetted out for the preparation of the pH 5 enzymes. The last of the fluid was sucked off and discarded, and the inner walls of the centrifuge tubes were carefully wiped dry with filter paper before the microsome pellets were transferred to a homogenizer tube. The combined pellets were homogenized with Medium A to give a suspension containing about 20 mg. of protein per ml. Microsomes prepared in this way have a very low content of acid-soluble nucleotides, as estimated by the ultraviolet absorption at 257 mμ of extracts made with ice-cold 0.3 N $HClO_4$. The dilution before centrifugation reduces the contamination by the soluble cell fraction, which has a high concentration of acid-soluble nucleotides.

To prepare the pH 5 enzymes, the supernatant fluid from the centrifugation at 105,000 × *g* was diluted with an equal volume of Medium B at 0° and the pH carefully adjusted to 5.2 by adding N acetic acid slowly with constant stirring. The solution was kept at 0° during this procedure. (A dilute mineral acid such as 0.1 N HCl can be used here, but the yield of pH 5 enzymes is lower, and some denatured proteins are precipitated along

with the active proteins.) The precipitate was collected by centrifugation at 0°, and washed once by suspension in Medium B, followed by centrifugation. The protein pellets were then dissolved in Medium A to give a solution of the pH 5 enzymes containing about 8 mg. of protein per ml. The pH 5 enzymes retain activity for several weeks at −10°.

Incorporation Experiments with pH 5 Enzymes-Microsome System—For incorporation of leucine-C^{14} into proteins, the incubation mixture contained, in a final volume of 1.0 ml., 0.3 ml. of microsome suspension (about 6 mg. of protein), 0.2 ml. of pH 5 enzymes solution (about 1.5 mg. of protein), 0.1 μmole of L-leucine-C^{14} (82,000 c.p.m.), 10 μmoles of PEP, 0.02 mg. of pyruvate kinase, 1.0 μmole of ATP, and 0.25 μmole of GDP or GTP. The

TABLE I

Requirements for Formation of Labeled Protein in pH 5 Enzymes-Microsome System

	C.p.m. per mg. protein
Complete system	167, 171
" " minus microsomes*	2
" " " pH 5 enzymes*	16
" " " pyruvate kinase	10
" " " PEP	13
" " " ATP	2
" " " GDP	25

The complete system contained microsomes, pH 5 enzymes, 0.03 mg. of pyruvate kinase, 0.1 μmole of L-leucine-C^{14} (82,000 c.p.m.), 10 μmoles of PEP, 0.5 μmole of ATP, and 0.25 μmole of GDP in a final volume of 1.0 ml.; incubation, 10 minutes at 37°. The final volume of all the flasks was brought to 1.0 ml. by the addition of 0.15 M KCl.

* Added after the incubation to give the same amount of protein, 11.5 mg., in all the flasks for the C^{14} assay.

mixture was incubated under 5 per cent CO_2—95 per cent N_2 for 10 minutes at 37°.

After the incubation the protein was precipitated, washed, and assayed for C^{14} with a thin window Geiger-Müller tube (counting efficiency about 12 per cent) and scaler, essentially as described previously (15).

Results

Conditions for Incorporation in pH 5 Enzymes-Microsome System—In Table I is found the effect on the incorporation of leucine-C^{14} into proteins caused by the omission of any one of the above constituents from the incubation mixture. There is no incorporation in the absence of microsomes. In the absence of pH 5 enzymes there is a low level of activity. This may result from residual contamination of the microsomes with the

pH 5 enzymes. The optimal amount of pH 5 enzymes protein was shown in other experiments to be about one-fourth that of the microsome protein. The pH 5 enzymes appear to be approximately four times as active as the whole soluble cell protein.

Either GDP or GTP is essential for the formation of labeled proteins in addition to ATP and a system for the generation of ATP. GDP was used in most experiments because it became available earlier. On the addition of GDP to give the "complete system" (Table I), the labeling of the proteins is as high as that obtained previously in the crude system (2). The concentration of GDP required for maximal labeling of proteins is shown in Fig. 1 to be approximately 0.15 μmole per ml. We have found in the

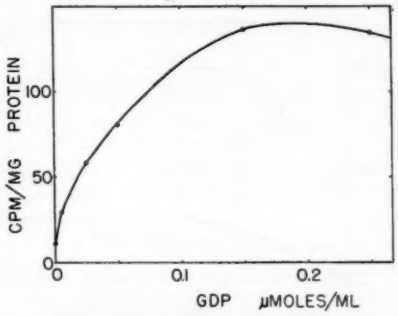

FIG. 1. The effect of GDP concentration on the formation of labeled proteins. Each flask contained microsomes (5.7 mg. of protein), pH 5 enzymes (3.3 mg. of protein), 0.1 μmole of L-leucine-C^{14} (82,000 c.p.m.), 10 μmoles of PEP, 0.02 mg. of pyruvate kinase, 0.5 μmole of ATP, 0.4 mg. of nucleoside diphosphate kinase preparation, in a final volume of 1.0 ml. Incubation, 10 minutes at 37°.

pH 5 enzymes some nucleosidediphosphatase (16) which hydrolyzes GDP to guanosine-5′-phosphate. The amount of GDP required for maximal incorporation would probably be lower in the absence of this enzyme.

With leucine-C^{14}, the protein labeling in the absence of added GDP has usually been from 5 to 15 per cent of that in the presence of GDP. Since the amount of GDP required is small, it seems reasonable to suppose that the low incorporation in the absence of added GDP is due to residual GDP and GTP in the microsomes and pH 5 enzymes. It has been shown by Schmitz *et al.* (17) that there is a considerable amount of GDP and GTP in rat liver.

For the formation of labeled proteins it is not enough to supply GTP, or GDP and a system for the generation of GTP from GDP; ATP and an ATP-generating system are required in addition. GTP in concentrations up to 3 μmoles per ml. has been found to be entirely inactive when tested

with microsomes, pH 5 enzymes, and leucine-C^{14}. There is no formation of labeled protein in the complete system minus ATP (Table I), though GDP, PEP, and pyruvate kinase are present. Pyruvate kinase can catalyze the transfer of phosphate from PEP to GDP to form GTP (18). There was no formation of labeled protein in another experiment when GDP, succinyl coenzyme A (10 μmoles per ml.), inorganic phosphate, and the GDP phosphorylation enzyme (7, 10) were added for the generation of GTP. These results all point to a requirement for ATP in addition to GDP or GTP.

The amount of ATP required is considerably greater than the amount of GDP required; maximal labeling of protein is obtained with 1.0 μmole of ATP per ml. (Fig. 2).

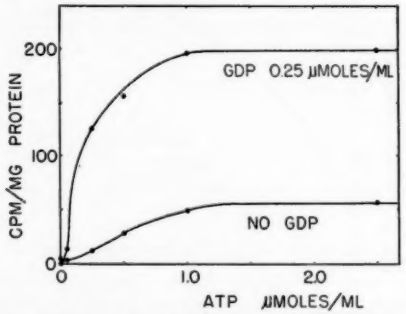

FIG. 2. The effect on formation of labeled proteins of increasing ATP concentration with and without added GDP. Each flask contained microsomes, pH 5 enzymes, 0.1 μmole of L-leucine-C^{14} (82,000 c.p.m.), 10 μmoles of PEP, 0.02 mg. of pyruvate kinase, in a final volume of 1.0 ml. Incubation, 10 minutes at 37°.

Several different systems for the generation of ATP, including the recently discovered carbamyl phosphate-carbamate kinase system (11), have been tested in the amino acid incorporation system. In each case GDP is required for active incorporation (Table II). ATP is also required with each of these systems.

Incorporation of Six Labeled Amino Acids—In all the experiments described up to this point, L-leucine-C^{14} was used. Other C^{14}-amino acids have been tested, and they are all actively incorporated into proteins in the present system when GDP is added (Table III). Because the specific activity of the six C^{14}-amino acids used here varied widely, the results in Table III have been given in millimicromoles of C^{14}-amino acid incorporated per gm. of protein during the 10 minute incubation. The labeling of proteins with these six amino acids in the absence of GDP is 3 to 15 per cent of that in the presence of GDP. For comparison with the present pH 5 enzymes-microsome system, the incorporation of five of the labeled

amino acids into proteins in the unfractionated system previously described (2) is also given in Table III.

TABLE II

GDP in Formation of Labeled Proteins with Different ATP-Generating Systems

The values are given in counts per minute per mg. of protein.

	No guanosine diphosphate	Guanosine diphosphate, 0.25 μmole
Phosphoenolpyruvate, 10 μmoles, pyruvate kinase, 0.03 mg.	13	172
Phosphocreatine, 20 μmoles, creatine kinase, 0.01 mg.	10	154
Carbamyl phosphate, 12 μmoles, *S. faecalis* R extract*	22	114

Each flask contained microsomes (4.8 mg. of protein), pH 5 enzymes (3.7 mg. of protein), 0.5 μmole of ATP, and 0.1 μmole of L-leucine-C^{14} (82,000 c.p.m.) in a final volume of 1.0 ml.; incubation, 10 minutes at 37°.

* A crude *S. faecalis* R extract containing carbamate kinase (11). It had been treated with Dowex 1 × 10 chloride to remove nucleotides.

TABLE III

Effect of GDP on Labeling of Proteins with Six C^{14}-Amino Acids

The values are given in millimicromoles incorporated per gm. of protein.

C^{14}-Amino acid		Unfractionated system	pH 5 enzymes-microsome system	
			No GDP	0.25 μmole of GDP
	μmole per ml.			
Glycine	0.10	25	7	67
DL-Alanine	0.25		10	79
L-Valine	0.10	88	3	90
L-Leucine	0.10	107	11	184
L-Isoleucine	0.10	104	7	85
DL-Phenylalanine	0.25	50	7	46

Unfractionated system: 0.7 ml. of supernatant fluid from centrifugation of homogenate at 15,000 × *g* (16 mg. of protein), 20 μmoles of phosphocreatine, in a final volume of 1.0 ml. pH 5 enzymes-microsome system: microsomes (3.7 mg. of protein), pH 5 enzymes (1.7 mg. of protein), 0.01 mg. of creatine kinase, 20 μmoles of phosphocreatine, 1.0 μmole of ATP, in a final volume of 1.0 ml.; incubation, 10 minutes at 37°.

Specificity of Guanosine Nucleotide Effect—GTP and GDP are equally active in stimulating the formation of labeled protein (Table IV). This raises the question as to which is the active compound in this system. We

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have found that there is in the pH 5 enzymes some nucleoside diphosphate kinase (7, 10) which catalyzes the following reaction: GDP + ATP ⇄ GTP + ADP. Thus both GDP and GTP would be present when either is added, but GTP would probably predominate, since there is a system for regeneration of ATP from ADP in the incubation mixture. In addition to this, when the PEP-pyruvate kinase system is used, the phosphate of PEP can be transferred directly to GDP to form GTP (18). If GTP is the active compound in the formation of labeled protein, then it is gener-

TABLE IV

Specificity of GDP and GTP in Formation of Labeled Proteins

Non-adenine nucleotides (0.25 μmole of each)	C.p.m. per mg. protein
None	17
Guanosine triphosphate	175
" diphosphate	158
Inosine triphosphate*	32
" diphosphate	33
Inosine-5′-phosphate	22
Cytidine triphosphate	11
" diphosphate	13
Cytidine-5′-phosphate	17
Uridine triphosphate	19
" diphosphate	13
Uridine-5′-phosphate	19
All 4 triphosphates	152

Each flask contained microsomes (5.0 mg. of protein), pH 5 enzymes (1.5 mg. of protein), 0.02 mg. of pyruvate kinase, 10 μmoles of PEP, 0.5 μmole of ATP, 0.1 μmole of L-leucine-C^{14} (82,000 c.p.m.), in a final volume of 1.0 ml.; incubation, 10 minutes at 37°.

* The net effect of inosine triphosphate is 15 c.p.m. per mg. of protein after subtracting the control value of 17.

ated at a sufficient rate by these reactions, since the addition of a nucleoside diphosphate kinase preparation to a complete system does not stimulate the incorporation. It has also been found that the nucleoside diphosphate kinase preparation cannot replace the pH 5 enzymes in the complete system.

Inosine diphosphate and triphosphate show some activity, but only about one-tenth that of GDP or GTP at the concentration tested (Table IV). This is interesting, since, in the case of the GDP phosphorylation enzyme, inosine diphosphate has about one-half the activity of GDP (7), and, in the case of oxalacetic carboxylase, inosine diphosphate has up to two-thirds the activity of GDP (19).

The pyrimidine nucleotides tested, cytidine and uridine mono-, di-, and triphosphates, are completely inactive (Table IV). When all the nucleoside triphosphates are combined, the labeling of the protein is no greater than with ATP plus GTP (Table IV).

Guanosine-5′-phosphate gives a small but definite stimulation of protein labeling (Table V). This could be due to conversion to GDP by a nucleoside monophosphate kinase (20). Guanosine diphosphate mannose (13), guanosine-3′-phosphate, and xanthosine diphosphate are all inactive (Table V).

Effect of Concentration of C^{14}-Amino Acids—In the earlier work on the unfractionated system it was found that, with increasing amounts of a C^{14}-

TABLE V

Relative Activity of Compounds Related to GTP in Formation of Labeled Proteins

The values are given in counts per minute per mg. of protein.

Non-adenine nucleotides (0.15 μmole)	No adenosine triphosphate	Adenosine triphosphate, 0.5 μmole
None	2	18
Guanosine triphosphate	4	144
Guanosine-5′-phosphate	1	60
Guanosine-3′-phosphate	2	13
Guanosine diphosphate mannose	3	19
Xanthosine diphosphate*	4	27

Each flask contained microsomes (4.4 mg. of protein), pH 5 enzymes (1.2 mg. of protein), 0.02 mg. of pyruvate kinase, 10 μmoles of PEP, 0.2 μmole of L-leucine-C^{14} (164,000 c.p.m.), in a final volume of 1.0 ml.; incubation, 10 minutes at 37°.

* The very low activity could be due to a trace of GDP, since it was prepared from GDP by treatment with HNO_2.

amino acid, a saturation concentration was reached above which there was no further increase in the labeling of the proteins (2). The specific activity of small amounts of a C^{14}-amino acid would be reduced by the endogenous amino acid of the tissue extract. This effect becomes insignificant when relatively large amounts of the C^{14}-amino acid are used. In the unfractionated system the saturation concentration of labeled alanine was very high, 2.5 μmoles of DL-alanine-C^{14} per ml., and the concentration of free alanine is known to be high in liver (21). In the pH 5 enzymes-microsome system the amount of endogenous amino acids has been reduced, and the saturation level of alanine is also reduced to 0.25 μmole of DL-alanine-C^{14} per ml. The leucine saturation level is nearly the same in the two systems, about 0.10 μmole of L-leucine-C^{14} per ml. The valine saturation level is low in the present system, 0.02 μmole of L-valine-C^{14} per ml. (Fig. 3).

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So far it has not been possible to stimulate the incorporation of a labeled amino acid by the addition of all the other commonly occurring amino acids. When, in addition to the C^{14}-amino acid, an equimolar mixture of the remaining seventeen L-amino acids is added at 0.01 to 0.25 μmole of each per ml., there is no stimulation of the incorporation of the C^{14}-amino acid. Neither is there stimulation by the further addition of glutamine and asparagine to give a total of twenty amino acids. It is possible that this lack of stimulation on adding amino acids is due to residual small amounts of endogenous amino acids in the liver cell fractions. It has been shown that the incorporation in this cell-free liver system is not a reversible process even when only a single amino acid, *i.e.* the labeled one, is added

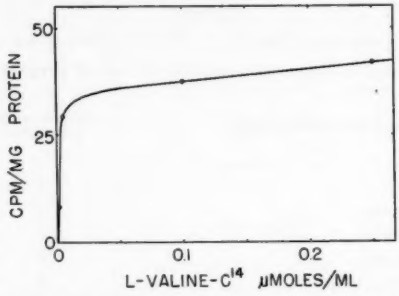

FIG. 3. The effect of L-valine-C^{14} concentration on the labeling of proteins. Each flask contained microsomes (4.5 mg. of protein), pH 5 enzymes (1.0 mg. of protein), 10 μmoles of PEP, 0.02 mg. of pyruvate kinase, 0.5 μmole of ATP, 0.15 μmole of GDP, and 0.4 mg. of nucleoside diphosphate kinase preparation, in a final volume of 1.0 ml. Incubation 10 minutes at 37°. The valine-C^{14} had a specific activity of 5.9×10^5 c.p.m. per μmole.

(4). This is in contrast to the reversible incorporation of certain labeled amino acids in the disrupted staphylococcal system in the absence of other added amino acids (22).

Partial Hydrolysis of Labeled Protein to Give Labeled Peptides—In the earlier experiments (2, 23) microsome protein labeled with leucine-C^{14} was hydrolyzed with 2 N HCl at 100° for different lengths of time, and the partial hydrolysates were analyzed by starch column chromatography (24). The specific radioactivity of the leucine released during the course of the hydrolysis was found to be nearly constant. On complete hydrolysis, 95 per cent or more of the C^{14} in the protein was recovered as leucine-C^{14}. In addition, the starch column chromatograms of the partial hydrolysates showed a large number of radioactive peptide-like peaks, and one C^{14}-peptide was tentatively identified as containing leucine and valine (2). This

was taken as an indication of the formation of α-peptide bonds in the incorporation of leucine-C^{14} into proteins.

It seemed desirable to repeat this type of analysis on the C^{14}-protein obtained in the pH 5 enzymes-microsome system. A large scale incorporation experiment was run with an incubation mixture like the complete system in Table I, except that the specific activity of the L-leucine-C^{14} was five times as high. This gave 218 mg. of protein, with 1224 c.p.m. per mg. This protein was hydrolyzed for 5 days at 50° in 12 N HCl in order to avoid the rearrangement of peptides which occurs in dilute acid at 100° (25). About one-third of the leucine-C^{14} of the protein was released. The starch column chromatogram of this partial hydrolysate again showed a number

TABLE VI

Chromatogram of Partial Hydrolysate of Protein Labeled with Leucine-C^{14}

	Effluent volume at peak	Per cent of total C^{14}
	ml.	
Large peptides containing leucine-C^{14}	52	14
Small peptides of leucine-C^{14}, isoleucine, and valine	162	20
Leucine-C^{14}	194	35
Unknown peptides containing leucine-C^{14}	217	8
" " " "	280	11
" " " "	307	3
Recovery		91

The leucine-C^{14}-labeled protein was prepared and hydrolyzed as described in the text. After removal of the HCl, the sample was put on a starch column (80 gm. of anhydrous starch, 2.2 cm. diameter) and eluted with *n*-butanol-15 per cent water (24).

of peaks of radioactivity and ninhydrin-reacting material at points where none of the known amino acids appear. The amount of C^{14} in these peaks and in the leucine peak is presented in Table VI. The first broad irregular peak which begins with the solvent front is apparently a mixture of large peptides (26). The second peak was resolved on a Dowex 50 column (27) into two radioactive peaks in addition to some leucine-C^{14}. The larger of the two peaks was hydrolyzed and analyzed for amino acids by paper chromatography. It was found to be a peptide or peptides containing isoleucine and valine in addition to leucine-C^{14}. This is consistent with the observations that peptides containing valine and isoleucine are resistant to hydrolysis (28, 29).

In a control experiment, leucine-C^{14} was added to some non-radioactive microsome protein which was then partially hydrolyzed with 12 N HCl as

above. The starch column chromatogram of the partial hydrolysate showed the same peaks of ninhydrin-reacting material, but there was no radioactivity, except of course in the leucine peak.

These results along with the previous ones provide evidence that the incorporation of leucine-C^{14} occurs by α-peptide bond formation and therefore that the reactions studied here could be steps in the normal process of protein synthesis.

DISCUSSION

The formation of labeled protein in this cell-free liver system appears to involve two main steps, the first being the carboxyl activation of amino acids catalyzed by soluble enzymes (5, 6) and the second the condensation of the carboxyl-activated amino acids to form polypeptide chains in the ribonucleoprotein particles of the microsomes (4). With regard to this second step, it was found that, after incorporation of labeled amino acids in this cell-free liver system, the ribonucleoprotein particles contained a large part of the total labeled protein. The same was found for the incorporation in rat liver *in vivo* at the earliest practical time intervals, that is 2 to 3 minutes after the intravenous injection of a labeled amino acid. Because of the similarity in distribution of labeled protein in these two cases, it was suggested that the incorporation in the cell-free system may represent the early stage in the process of protein synthesis as it occurs in the intact cell.

The first step in the formation of labeled protein appears to be the carboxyl activation of amino acids, as was first proposed by Lipmann (30). The soluble fraction of liver, which is essential for incorporation of labeled amino acids into proteins, contains enzymes which catalyze the activation of specific amino acids by the following type of mechanism (5): enzyme + ATP + L-leucine $\rightleftharpoons$ enzyme(L-leucyl $\sim$ AMP) + pyrophosphate. These activating enzymes are concentrated in the pH 5 enzymes (6) which are found in the present paper to be essential for incorporation. Other evidence that amino acid-activating enzymes are involved in the formation of labeled proteins has also been reported (6). The loss of amino acid-activating activity in preparations of pH 5 enzymes stored at $-10°$ parallels loss of activity for incorporation as far as this has been tested. When increasing concentrations of hydroxylamine are added to the activating system, the yield of aminohydroxamic acid increases to a maximum at 1.2 M hydroxylamine. Increasing concentrations of hydroxylamine added to the incorporation system cause a roughly parallel inhibition of the incorporation of leucine-C^{14} into protein. Thus all the evidence obtained so far points to activation of the C-14 amino acid by the soluble enzymes as a step in incorporation. Work is in progress to obtain more direct evidence by fractionating the pH 5 enzymes and testing the fractions in the

incorporation system described in this paper. These experiments will also inquire as to whether other soluble enzymes are necessary for incorporation.

So far there is no direct evidence on the mechanism of action of GDP or GTP in the formation of labeled proteins. It appears that ATP, and not GTP, is involved in carboxyl activation of amino acids (6). A possible rôle for GDP or GTP is suggested by the fact that the amino acyl ~ AMP is not released from the activating enzyme (5, 6). GDP or GTP might serve as an acceptor for the amino acyl group from the enzyme(amino acyl ~ AMP) and then as a donor of the amino acyl group in the elongation of the polypeptide chain in the ribonucleoprotein particles of the microsomes. The preparation of various amino acyl derivatives of GDP and GTP would make possible a test of this hypothesis.

Another possibility is that GDP or GTP may participate in the formation of a complex guanosine-containing nucleotide analogous to those described by Cabib and Leloir (13) and Binkley *et al.* (31) and that the complex nucleotide would be the actual cofactor in the incorporation reaction. It is of interest that the nucleotides isolated by Binkley *et al.* contain peptides and have peptidase activity. Binkley has proposed that they may be involved in protein synthesis (32).

It is perhaps desirable to emphasize the difference between the effect of GDP or GTP reported here and the nucleotide effects described by Gale and Folkes (33). GDP or GTP in the liver system increases the incorporation of all six labeled amino acids so far tested about 10-fold. This incorporation is primarily into the ribonucleoprotein particles of the microsome fraction (4). There is no incorporation if the ribonucleic acid-containing microsomes are omitted, and it appears that the ribonucleic acid must be intact, since ribonuclease at low concentrations abolishes the incorporation (2). Thus, in the liver system, the effect of GDP or GTP requires the presence of the ribonucleic acid. In disrupted staphylococci, on the other hand, Gale and Folkes report that the incorporation of a given amino acid into protein is stimulated by a particular fraction from ribonucleic acid which has been digested with ribonuclease. This stimulation occurs only if the nucleic acid has first been partially extracted from the disrupted staphylococci.

SUMMARY

For the incorporation of labeled amino acids into proteins in an enzyme system from rat liver, two cell fractions, the microsomes and the soluble cell fraction, are essential in addition to the C^{14}-amino acid, ATP, and an ATP-generating system. When the active proteins of the soluble cell fraction are freed of low molecular weight substances by a precipitation

at pH 5, there is little formation of labeled protein unless either guanosine diphosphate or triphosphate is added. Either of these nucleotides can restore the activity to the level of the crude system. The incorporation into proteins of each of the six C^{14}-amino acids so far tested requires the addition of guanosine di- or triphosphate. Inosine di- and triphosphate have only about one-tenth the activity of the guanosine nucleotides. Cytidine and uridine di- and triphosphates, xanthosine diphosphate, and guanosine diphosphate mannose are all inactive.

Guanosine triphosphate in amounts up to 3 μmoles per ml. does not stimulate the formation of labeled proteins in the absence of ATP; ATP and an ATP-generating system are required in addition to guanosine di- or triphosphate.

The microsome fraction, which is required in this system, contains the cytoplasmic ribonucleic acid. The effect of the guanosine di- or triphosphate occurs in the presence of this ribonucleic acid.

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SOME MOLECULAR AND KINETIC PROPERTIES OF HEART MALIC DEHYDROGENASE*

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Although malic dehydrogenase[1] was discovered in 1910 (1, 2) and several methods for purification of the enzyme have appeared (3–6), the molecular properties of this important catalyst in the tricarboxylic acid cycle have not previously been described. The present report gives an account of the isolation of the enzyme from heart muscle in a state of purity which has permitted the measurement of such properties.

EXPERIMENTAL

Isolation of Malic Dehydrogenase

Measurement of Activity—The measurements of activity performed in conjunction with the isolation of MDH were carried out by measuring the rate of reduction of DPN at high concentrations of the coenzyme and substrate, L-malate.

The sensitive recording spectrophotometer, although similar to that used earlier with lactic dehydrogenase, has not been previously described in detail. The apparatus consisted of a Beckman model DUR monochromator, attached through a specially constructed amplifier to a 50 mv. strip chart recorder with a 2 second pen speed. The amplifier was equipped with a voltage divider and "bucking" circuit to give a choice of either 0 to ∞ or 0 to 0.0458 optical density full scale (27 cm. chart paper). The more sensitive scale, used exclusively in the present work, permits an accurate reading of optical density to the fourth decimal place. The apparatus is thus essentially similar to that used by Bock and Alberty (7) with fumarase, except that our sensitive scale has been expanded by an additional factor of 2.

For the enzymatic assays, spectrophotometric cuvettes of 1 ml. capacity were charged with 0.9 ml. of 0.1 M glycine-NaOH buffer, pH 10, 0.05 ml.

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[1] The following abbreviations are used: MDH, malic dehydrogenase; DPN, oxidized diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

XUM

of 5×10^{-2} M DPN (Pabst), and 0.05 ml. of 2.0 M recrystallized sodium L-malate (Eastman). These conditions will hereafter be referred to as the "assay system." The spectrophotometer was balanced at 340 mμ, and the reaction was initiated by adding the enzyme in a volume of 0.05 ml. on the tip of a glass scoop. The temperature of the cuvette was maintained at 25° by a circulating thermostat.

The yield of MDH during the isolation procedure was expressed as a unit, which corresponded to the reduction of 1 mμmole of DPN per minute under the above conditions. The yield of total protein was followed by measuring the optical density at 280 mμ after application of a correction for excessive density at 260 mμ (8). An optical density of 1.0 $cm.^{-1}$ was arbitrarily chosen to represent 1.0 mg. of protein per ml. as a first approximation.

Fractionation Steps—Unless otherwise stated, all isolation steps were performed at 4°.

Thirty fresh pig hearts were chilled in cracked ice and freed from fat and connective tissue. After passage through a power-driven meat grinder, the tissue was washed successively five times with 25 liter batches of ice water, strained through cheese-cloth, and then treated with 15 liters of cold 90 per cent acetone-10 per cent water. After 30 minutes in the aqueous acetone, the tissue was treated twice with successive 10 liter batches of pure acetone. The extracted tissue was collected with cheese-cloth, placed in a large desiccator, and the solvent removed with an oil pump.

The MDH was extracted from the acetone powder with 10 liters of 0.05 M sodium phosphate buffer, pH 7.0. The extraction was carried out over an 8 minute period in a 1 liter Waring blendor, which was run for 1 minute intervals to avoid overheating. The insoluble débris was removed in a refrigerated 13 liter centrifuge to give a milky solution of the enzyme.

The preparation was brought to 0.3 saturation with ammonium sulfate by addition of 176 gm. of the salt per liter of extract. After 12 hours at 4° the suspension was centrifuged and the precipitate discarded. The clear, amber-brown supernatant liquid was brought to 0.5 saturation by the addition of a further 125 gm. of ammonium sulfate per liter of original solution. After 12 hours at 4° the precipitate was removed and discarded as before. Finally, 100 gm. of ammonium sulfate were added per liter of original solution to give a saturation of 0.65, and after 12 hours at 4° the precipitate was removed by filtration and retained. The filtrate was discarded.

The protein precipitate obtained as above was dissolved in the least volume, usually 250 to 300 ml., of 0.01 M maleate buffer, pH 6.2, which was also 0.2 M with respect to NaCl. The preparation was dialyzed against the maleate buffer for about 15 hours and then against fresh maleate buffer containing 10^{-4} M zinc chloride. Dialysis against maleate buffer serves to re-

move phosphate; the latter would be objectionable since it would precipitate the zinc ion. After a 12 hour dialysis, the precipitated protein was removed by centrifugation and discarded.

The amber-colored solution of the enzyme was next fractionated with ethanol, which was added through a sintered glass filter. During the addition of ethanol the enzyme solution was agitated vigorously with a mechanical stirrer. When the concentration had exceeded about 10 per cent, further additions of ethanol were made at $-10°$. The precipitate appearing at 40 per cent ethanol was centrifuged at $-10°$, and the supernatant fluid was brought to 50 per cent ethanol. The precipitate at this stage contains some MDH, but attempts to carry it through to the final isolation have not been successful. The fractionation was continued, and the precipitates at 60 and 70 per cent ethanol were collected and assayed separately. The

TABLE I

Yield and Purity of Malic Dehydrogenase at Various Purification Stages

	Total enzyme	Total protein*	Turnover No.	Fraction activity recovered
	μmoles	*gm.*	*moles DPN 40,000 gm. protein^{-1} min.$^{-1}$*	
Phosphate buffer extract....	67.5	100	260	1.0
Ammonium sulfate ppt.....	60.0	31	700	0.9
Aqueous zinc supernatant...	27.5	11	880	0.4
Ethanol zinc ppt., 70%.....	6.5	0.26	9700	0.1

* Based on an optical density of 0.46 mg.$^{-1}$ ml.$^{-1}$.

purest enzyme is contained in the 70 per cent ethanol fraction; however, if the 60 per cent fraction has been found to be only slightly less active, the two fractions are combined and dissolved at once in cold 0.05 M phosphate buffer, pH 7.0.

The enzyme solution now contains only very minor components such as traces of cytochrome *c*, which were removed by batchwise treatment with carboxymethylcellulose (9). The remaining minor impurities all have a much higher negative electrophoretic mobility in neutral phosphate buffer and hence the MDH may be recovered from the cathodic limb. Final isolation of the enzyme was achieved in electrophoretic experiments with the Perkin-Elmer model No. 38A apparatus.

Table I summarizes the yield and purity of MDH at the major purification steps. The unit of activity here has been replaced by the turnover number and the protein measurements were corrected on the basis of an optical density of 0.46 mg.$^{-1}$ ml.$^{-1}$ at 280 mμ.

The activity of an extensively purified preparation of MDH was found

to remain stable at room temperature in neutral buffers for days. The slight decrease in activity which occurred with time could be prevented by keeping the enzyme under N_2 or by prior treatment with cysteine. However, once the activity had been lost through aging, it could not be restored by treatment with cysteine.

Criteria of Purity—In 0.01 M maleate and 0.1 M phosphate buffers, pH 6.3 and 7.0, respectively, the isolated enzyme showed only a single boundary in the ultracentrifuge. Similarly, electrophoresis experiments in these buffers at 100 volts and 10 ma. in the 2 ml. cell for 10 hours did not reveal the presence of impurities. Preparations which have met these criteria have consistently given a turnover number of 9700 moles of DPN per mole^{-1} MDH min.$^{-1}$ in the assay system.

All attempts to crystallize the enzyme have been unsuccessful.

Molecular Properties of Malic Dehydrogenase

Extinction Coefficient—Insufficient quantities of isolated MDH were obtained for a determination of the extinction coefficient based on dry weight. However, it was possible to measure this coefficient by two alternative, independent methods. In the first of these an optical density and total nitrogen analysis (10) were performed on solutions of MDH in 0.1 M phosphate buffer, pH 7.0, which had been removed from the electrophoresis apparatus. Assuming a nitrogen content of 16 per cent, this method gave optical density values of 0.408, 0.504, and 0.492 (average 0.46) for 1.0 mg. of MDH ml.$^{-1}$ cm.$^{-1}$ at 280 mμ. In the second method, the area of an electrophoretic pattern given by a known concentration of bovine serum albumin was compared with the MDH patterns. In duplicate analysis the latter procedure gave optical density figures of 0.48 and 0.44 (average 0.46) for 1.0 mg. of MDH ml.$^{-1}$ cm.$^{-1}$ at 280 mμ.

The optical density ratio, 280:260 mμ, was consistently found to be 1.4. On the basis of a molecular weight of 40,000, the molar extinction coefficient at 280 mμ is 1.84×10^4. In all quantitative work with isolated MDH, the enzyme concentration was established by optical density measurements at 280 mμ.

Electrophoretic Mobility—Quantitative measurements of the electrophoretic mobility of MDH have not been made. Qualitative experiments in 0.01 M maleate buffers would place the isoelectric point between pH 6.1 and 6.4.

Diffusion Coefficient—The diffusion coefficient, $D_{20,w}$, was measured in 0.1 M phosphate buffer, pH 7.0, with the 2 ml. Perkin-Elmer electrophoresis cell. The MDH concentration was about 5 mg. ml.$^{-1}$ and the temperature 2°. The boundary was sharpened with a capillary needle at the start of the experiment. The height-area method and the extrapolation to zero

time were used in calculation of $D_{20,w}$. A value of 8.47×10^{-7} cm. sq. sec.$^{-1}$ was obtained.

Sedimentation Coefficient—The sedimentation velocity coefficient, s_{20}, was measured in 0.01 M (0.015 ionic strength) maleate buffer, pH 6.25, with use of the synthetic boundary cell of the Spinco model E ultracentrifuge (11). The concentration dependence of s_{20} appeared to exhibit a slight positive slope (Fig. 1). The value of s_{20} extrapolated to zero protein concentration was 3.6×10^{-13} sec.

Molecular Weight—Assuming a partial specific volume of 0.74 ml. gm.$^{-1}$,

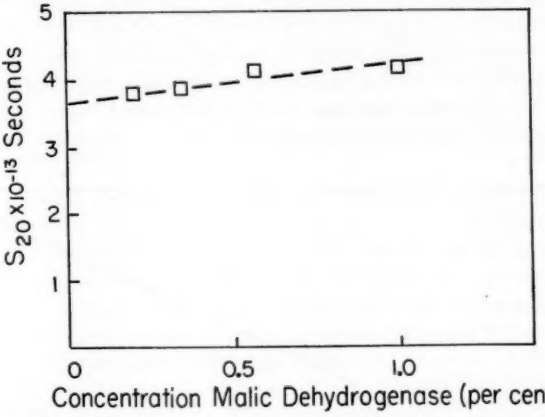

FIG. 1. Sedimentation coefficient, s_{20}, as a function of concentration for malic dehydrogenase.

the molecular weight from the sedimentation and diffusion data is 40,000 ± 5000.

Kinetic Properties of Malic Dehydrogenase

Turnover Number—The turnover numbers for the oxidation of L-malate at pH 10 and for the reduction of oxalacetate at a series of pH levels have been measured.

In the assay system (0.1 M L-malate, 2.5×10^{-3} M DPN, 0.1 M glycine-NaOH buffer, pH 10) the turnover number was 9700 moles of DPN mole^{-1} MDH min.$^{-1}$. Measurements of the rate of oxidation of L-malate at pH levels less than 10 have been avoided because of the unfavorable equilibrium condition which prevails at the lower pH. The reaction was found to exhibit an anomalous acceleration after the substrate concentration had exceeded about 10^{-4} M.

The variation in maximal initial velocity, V, with pH for the reduction

of oxalacetate is given in Table II. These data were obtained by use of the double reciprocal plots (12). The figures are seen to pass through a maximum, 44,000 moles of DPNH mole^{-1} MDH min.$^{-1}$, at pH 6.9.

TABLE II

Michaelis Constants and Maximal Initial Velocities for Oxidation of DPNH by MDH As Function of pH

pH	Buffer (0.1 ionic strength)	K_m	V ($\times 10^{-4}$ mole DPNH mole^{-1} MDH min.$^{-1}$)
		μmoles	
6.25	Maleate	6.8	3.5
6.9	Phosphate	27.8	4.4
7.4	"	4.2	3.8
7.9	Pyrophosphate	11.8	3.5
8.8	Glycine-NaOH	7.6	1.7

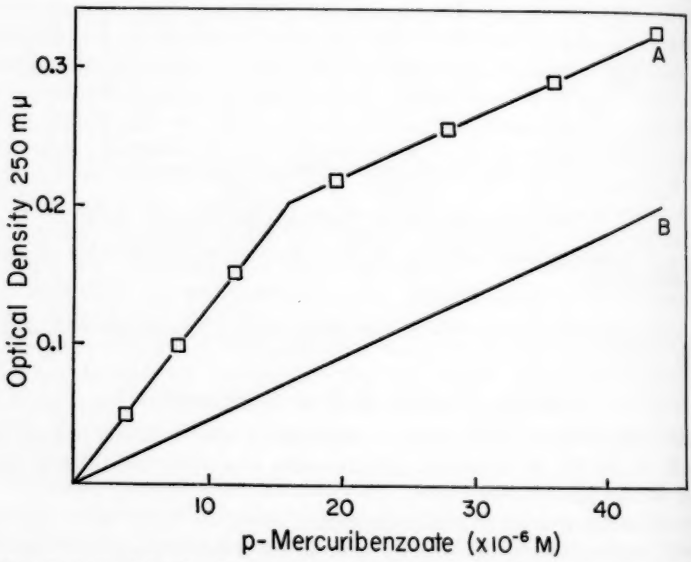

FIG. 2. Optical density increment at 250 mμ for *p*-mercuribenzoate alone (Curve B) and in the presence of malic dehydrogenase (Curve A).

Michaelis Constants—The Michaelis constant, K_m, for L-malate at pH 10 was measured by extrapolating the velocity measurements in dilute solution to the limit of $1/v = 0$. A figure of 5.5×10^{-5} M was found. The

reasons for giving more weight to the measurements made in dilute solution will be discussed below. The K_m for DPN has not been measured because of the uncertainty in the saturation level of L-malate.

Table II contains a summary of the Michaelis constants evaluated for DPNH in the pH range 6.25 to 8.8. The figures for the oxalacetate have not been obtained because of the instability of this compound.

Inhibition Studies—The number and reactivity of the sulfhydryl groups of MDH were measured by the spectrophotometric procedure of Boyer (13). The experiments consisted of titrating a 2.4×10^{-6} M solution of MDH with small aliquots of a 3.4×10^{-4} M solution of *p*-mercuribenzoate. The solvent used was 0.1 M phosphate buffer, pH 7.0, at 25°. Since the reaction of the mercurial with MDH was not instantaneous, a 10 minute equilibration period was allowed after each successive addition of inhibitor. Small aliquots of the reaction mixture were removed from time to time and tested for enzymatic activity in the assay system. The experimental data are shown in Fig. 2.

With the use of the optical density increment for the reaction of *p*-mercuribenzoate with cysteine, 7.29×10^{-3} mole^{-1}, values of 6.8 to 7.4 moles sulfhydryl mole^{-1} MDH were found. The measurements of activity revealed that complete suppression of the enzymatic activity was achieved only after all seven or more sulfhydryl groups in the enzyme had reacted with the mercurial. All the attempts to reactivate the inhibited enzyme with cysteine or glutathione were unsuccessful.

DISCUSSION

In the preparation of MDH, our chief departure from the methods of Straub (4) and Ochoa (6) is in the use of a zinc-ethanol fractionation step. Since we have obtained activity data under the identical conditions used by Ochoa (6) for an MDH preparation according to Straub (4), it is possible to estimate the purity of the Straub enzyme. The turnover number of the latter preparation has been given by Ochoa (6) as 35,000 moles of DPNH per 100,000 gm. of protein^{-1} min.$^{-1}$. On the basis of a molecular weight of 40,000 for MDH, this is a turnover number of 14,000 moles of DPNH mole^{-1} MDH min.$^{-1}$ at pH 7.4. Since the figure given in Table II under these conditions is 38,000, it may be concluded that the Straub preparation is of 40 per cent purity. Straub (4) found that his MDH was about as active on a weight basis as crystalline heart lactic dehydrogenase. Since the molecular turnover numbers of MDH and lactic dehydrogenase (14) are not very different, the activity ratio of these two enzymes is given by the ratio of their molecular weights, 135,000:40,000. Thus the preparation of MDH which we have obtained is somewhat over three times as active as crystalline heart lactic dehydrogenase on a weight basis.

MDH is unique among the known pyridine nucleotide dehydrogenases in having the lowest molecular weight. This property would be of obvious advantage if it were desired to investigate the structural chemistry of this protein, especially if the enzyme contained more than one active site.

It has not been possible to demonstrate a spectral shift on mixing MDH with DPNH, as has been done with both the liver alcohol (15) and heart lactic dehydrogenases (16).

Several explanations are possible for the abnormal increase in velocity of oxidation of L-malate at high levels of this substrate. The L-malate may be removing an inhibitor or there may be a cooperative phenomenon between more than one active site on the enzyme. The Michaelis constants reported by us for MDH were obtained by extrapolation of measurements made in dilute solution, since with fumarase (7) these were the only data which would fit the Haldane equation.

The maximal initial velocity of DPNH oxidation as a function of pH is of interest. The measurements indicate that two ionizable groups in the enzyme or enzyme substrate compound, one with a p$K'a$ of about 8.5, are required for activity. In an acid-strengthening field the sulfhydryl groups of cysteine may possess such a p$K'a$ (17).

The failure to reactivate MDH after treatment with mercuribenzoate is an observation which deserves comment. Alcohol dehydrogenase from fish liver is also apparently irreversibly inactivated with *p*-mercuribenzoate (18). On the other hand the *p*-mercuribenzoate derivative of lactic dehydrogenase, unlike MDH, is instantly and almost quantitatively reactivated with cysteine (19).

SUMMARY

1. Malic dehydrogenase has been isolated from pig heart muscle.

2. The molecular weight from sedimentation and diffusion measurements is 40,000 ± 5000.

3. The Michaelis constants and turnover numbers as a function of pH as well as other molecular properties of the enzyme have been established.

4. By use of the Boyer (13) technique, the enzyme has been found to contain about seven sulfhydryl groups per mole. The activity is not completely abolished until all the available sulfhydryl groups have been allowed to react with *p*-mercuribenzoate.

The authors wish to express their appreciation to Miss Jean Miller for performing the sedimentation analysis.

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A NEW MOIST CHAMBER RESPIROMETER FOR THE STUDY OF THE METABOLISM OF TISSUE SLICES IN VITRO*

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Deficiencies in the classical Warburg tissue respiration technique incident to the immersion and continuous agitation of thin tissue slices in a liquid medium have been long and generally recognized. Rodnight and McIlwain recently have described a modified technique (2), in which the gas exchange of the tissue slice is measured in non-aqueous media or under "moist" aqueous conditions with very small volumes of medium. In the case of some tissues, notably brain and kidney, they observed appreciably higher rates of oxygen uptake than with the usual Warburg procedure. In the course of isotopic tracer experiments on the metabolism of cytochrome *c in vitro* (1), we found that the recovery of the chromoprotein was very poor when slices of rat liver were incubated at 37° for 2 to 3 hours in appropriate aqueous media. Furthermore, the small amounts of cytochrome *c* which were isolated necessitated the use of relatively large quantities of radioactive isotopes of high specific activity. To overcome these difficulties, we have designed a new apparatus, based on the "moist tissue" principle (2), which has made possible the incubation of large numbers of tissue slices in comparatively small volumes of medium for periods of time longer than 6 hours, with maintenance of a steady state of oxygen consumption and excellent recoveries of cytochrome *c* at the termination of the incubation.

In this paper the new *moist chamber respirometer*, which should have many areas of usefulness in studies of metabolism *in vitro*, is described. Data are furnished from which the inference may be drawn that the metabolic performance of tissue slices in this "moist" method is maintained at an optimal level (as judged by the rate of oxygen consumption) 2

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to 3 times longer than in the conventional "wet" or immersion Warburg technique.

Equipment and Technique

Moist Chamber Respirometer—The new apparatus is illustrated in Fig. 1. The respirometer is made from a Corning Pyrex brand fritted glass Büchner funnel. The fritted glass plate is of coarse porosity (pore size of about 14 μ) and 9 cm. in diameter. Standard taper ground joints connect the U-tube manometer, M, and its adapter, A, to the modified funnel. The vessel readily accommodates fifteen slices of tissue, each of the size and thickness used in the conventional Warburg technique. Hence, about 3 gm. of tissue may be run in a single vessel in contrast with 200 mg. in the Warburg method. Also, it may be obvious that in the new equipment it is possible to study sections of large organs, such as tissue slices cut from a dog's brain. The glass tubing, with stop-cocks a, b, and c, is provided for gassing (see below). While we have adapted the stand of the apparatus (see legend to Fig. 1) for use in the constant temperature water bath provided for our Warburg equipment, an important feature of the new technique is that *no shaking of the vessels is required.* Hence, elaborate and costly shaking devices are unnecessary, in the event that one may contemplate the provision of a new constant temperature bath.

Calibration—The functional volume of the respirometers is of the order of 650 to 660 ml. This is obtained by weighing the vessels empty and then filled with water to a given point (Fig. 1) on the manometer. For this purpose a large sensitive balance was used. Since a simple U-tube manometer filled with Brodie's solution is employed, the expression for the vessel constant, K, is given by

$$K = (2V/RT) + (273a/22.4T)$$

where a is the cross-sectional area of the manometer (here = 6 mm.^{2}), and V, R, and T have the usual connotation. For the development of this equation reference may be made to Burk and Hobby (3). K for our apparatus is of the order of 5 μmoles per mm. of change in one arm of the manometer. (To obtain K in terms of micromoles, V is expressed in microliters and R = 821 μl. per mm. per μmole per degree absolute.)

Technique—In Step 1, the chamber is flushed with oxygen from below, the gas being admitted through the tube on the right (Fig. 1) and passed through the fritted glass disk. During this operation the manometer, M, and its adapter, A, are removed from the apparatus, stop-cock a is opened, and stop-cocks b and c are closed. In Step 2, stop-cock a is closed, and medium is applied to the fritted disk. 5 to 10 ml. of medium, depending on the porosity of the particular disk, are used. Stop-cock a is now partially opened, allowing the medium to become saturated with oxygen.

For Step 3, stop-cock *a* is closed and stop-cock *b* is opened. While the gas is flowing through the upper part of the chamber, the tissue slices are

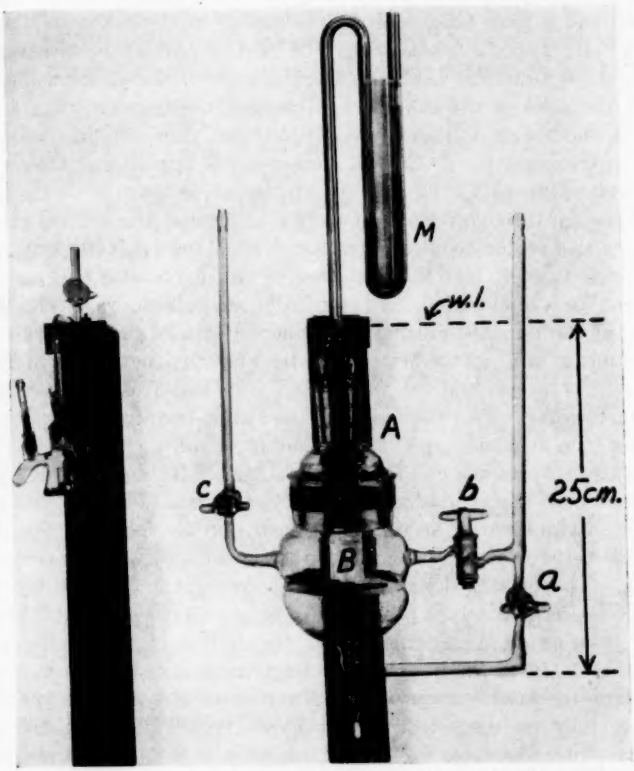

FIG. 1. Photograph of the moist chamber respirometer (right) and the conventional Warburg vessel (left). The apparatus, constructed from a Büchner funnel with a large, fritted glass disk of coarse porosity (see the text), is adapted to the stand originally fabricated for the Warburg vessel by means of a suitable stainless steel bracket and clamp. The vertical dimension of 25 cm. furnishes an index of the size. *w. l.* represents the water level in the constant temperature bath, the apparatus being immersed to above the bulb of the manometer, *M*. The calibrated volumes of the vessels, which include the bulb of the manometer (to level *w. l.*) and connecting glass tubing, are 650 to 660 ml. The fritted glass plate (9 cm. in diameter) accommodates, as shown, some fifteen slices of tissue and in its center a small glass beaker, *B*, with filter paper and alkali to take up CO_2. All joints are rendered water- and gas-tight by means of light Celvacene vacuum grease.

placed on the porous plate. Each slice is then carefully moistened with medium delivered by pipette, about 1 ml. sufficing for fifteen slices. In

most of our runs a total of about 7 ml. of medium was used for saturating the porous disk and covering the tissue slices. If measurement of oxygen uptake is to be performed, at this stage a 10 ml. beaker containing 3 ml. of alkali and a large filter paper roll is placed in the center of the disk (*B*, Fig. 1). The small beaker may be held in position by twisting two pipe cleaners around it below its lip; the ends of the cleaners then rest against the sides of the chamber. If oxygen consumption data are not required, the beaker and alkali may be omitted, thus affording additional space for tissue slices. In Step 4, stop-cock *c* is opened and the manometer, *empty of Brodie's fluid*, with its adapter, *A*, is attached to the chamber. Gassing, through open stop-cocks *b* and *c* and the unfilled manometer, is carried on for about 5 minutes. A small bore polyethylene tube is then inserted deeply into the open arm of the manometer and gassing is continued for a brief period. All stop-cocks are now closed and the gas is shut off at the tank, permitting the manometer to be filled with Brodie's fluid by means of a syringe attached to the polyethylene tube. In Step 5, stop-cock *c* is opened and the apparatus (on its stand) is immersed in the water thermostat. This stop-cock is closed after temperature equilibrium (37°) has been attained, a period of about 45 minutes.

Variants in Apparatus and Procedure—It should be obvious that smaller respirometers of similar design may be constructed. The chamber could be made in two separate sections with provision for clamping the fritted glass disk between them. Such an arrangement would be convenient in cleaning. The present design with the porous plate fused to the glass wall was chosen to avoid the possibility of streaming effects along the edges, thereby insuring an even diffusion and distribution of gas to all parts of the chamber. However, in this type of apparatus it is difficult to recover the medium for analytical purposes. If the latter is required, a variant in technique may be suggested. Small Pyrex brand, coarse fritted glass Gooch crucibles (diameter = 20 mm., capacity = 8 ml.) are arranged on the dry porous plate of the large chamber. Only the fritted plates of each crucible are wet with the medium. Each crucible accommodates a single slice of tissue. Otherwise, the steps in the procedure are identical with those described above. At the end of the run the crucibles are removed, and the medium may be readily recovered by washing. Also, individual crucibles may be removed during the course of the run.

RESULTS AND DISCUSSION

It is evident from the data supplied in Fig. 2 that in the new moist chamber respirometer the oxygen consumption of tissue slices (of brain, kidney, liver, and diaphragm) is maintained at nearly linear rates for at least 10 hours. Between the 6th and 10th hour the respiration rates measured were within 82 to 92 per cent of the earliest measurable values. Actually,

the rate of oxygen consumption is well maintained for considerably longer than 10 hours. In contrast, as shown in Fig. 2, the oxygen uptake of similar tissue slices in the conventional Warburg method, with the possible exception of diaphragm muscle, diminishes rapidly after the 3rd hour (or earlier) and is negligible between the 6th and 10th hour. It may be pointed out that the diaphragm is not sliced, but pieces of it are snipped out. Hence, there is far less traumatized surface exposed to leaching or

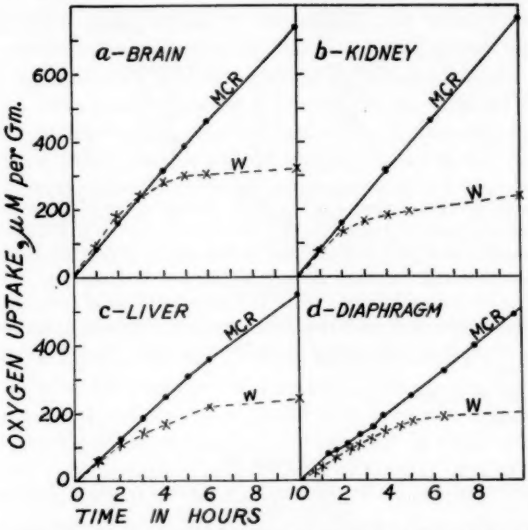

FIG. 2. Oxygen consumption in micromoles per gm. wet weight of tissue. The medium was 0.033 M phosphate buffer and 0.100 M KCl, pH 7.4. Solid line, *MCR*, 0.5 mm. thick slices in the moist chamber respirometer; broken line, *W*, 0.5 mm. thick slices with the conventional Warburg technique.

other effects by the medium during incubation. Even with this tissue, however, superior results are secured with the new respiration technique.

In the modified Rodnight and McIlwain technique (2), the length of time that a slice can continue to respire depends on the amount of substrate which can be made available to it. These investigators reported that slices laid on moist filter paper respired at rates 10 to 15 per cent lower than those observed when the tissue slices were supported on wire mesh frames. We ascribe the excellent maintenance of respiration in our apparatus to the fact that the fritted glass disk, wet with well oxygenated medium, allows a continuous supply of substrate to diffuse into the tissue slice through a relatively large contact area.

The physical appearance of the tissue slices after 6 hours in our apparatus

is remarkably good, especially in the case of liver. The excellent condition of the tissues after 6 hours is reflected by the fact that the extraction of cytochrome *c* from liver and kidney slices was equal to that obtained on aliquots before incubation (Table I). However, between the 6th and 10th

TABLE I

Cytochrome c Content (C) of Rat Tissue Slices Incubated in Vitro at 37°

Tissue	C* before incubation	C* after 3 hrs. in 5 volumes of medium	C* after 6 hrs. in moist chamber respirometer
	γ per gm.†	γ per gm.†	γ per gm.†
Kidney	272‡	43.3	212
Liver	110‡	44.3	134
		31.2	105
		39.3	116

* The amount of cytochrome *c* is based on the quantity of the purified pigment recovered from a column of Amberlite IRC-50 resin according to Palêus and Neilands (4) after extraction and preparation from the slices by a combination of the techniques of Rosenthal and Drabkin (5) and Keilin and Hartree (6). The estimation of cytochrome *c* by this technique (owing to incompleteness of recovery) gives lower values than those obtained with the Rosenthal and Drabkin analytical procedure (7, 8), which for rat liver and kidney were 178 and 346 γ per gm. wet weight of tissue, respectively.

† Wet weight of tissue.

‡ Mean values.

TABLE II

Water Content of Rat Tissue Slices after Varying Periods of Time in Moist Chamber Respirometer

Tissue	Time of incubation	Ratio, wet weight to dry weight		Water content	
		Initial	Final	Initial	Final
	hrs.			per cent	per cent
Brain	10	4.60	6.84	82.1	87.2
Kidney	10	4.46	6.35	81.7	86.4
Liver	3	3.65	3.99	78.5	80.0
"	10	3.49	5.55	77.7	84.7
Diaphragm	10	4.78	5.37	82.7	84.3

hour the tissues begin to swell visibly, and appreciable increases in their water content were found after 10 hours (Table II).

The new moist chamber respirometer offers several advantages over the conventional Warburg method and over modified techniques in which the Warburg vessels are utilized. It is thus possible to lengthen greatly the period of incubation, to pool large numbers of tissue slices, and at the same

time to decrease the volume of medium employed materially. These conditions are nearly ideal for experiments involving the measurement of the incorporation of isotopes into constituents of tissue slices *in vitro*.

Certain disadvantages of the present apparatus should be mentioned. Because of the volume of the vessel, temperature equilibrium and probably CO_2 diffusion equilibrium are not reached until about 45 minutes after immersion of the vessel in the water bath at 37°. Hence, the initial rates of oxygen consumption cannot be measured. An additional factor precludes an accurate measurement of initial rates of respiration, the time required, even with two persons working simultaneously, for the cutting of twenty to thirty tissue slices, which represent the capacity of the vessel. In spite of these limitations, as is evident in Fig. 2, the earliest measurable rates of oxygen uptake for the several tissues studied in the moist chamber respirometer were equal to the initial rates measured by the usual Warburg method. Improvements in procedure are currently being investigated and may appreciably shorten the early equilibration period.

SUMMARY

A new moist chamber respirometer has been described. In this apparatus the metabolism of fifteen to thirty slices of tissue may be effectively studied *in vitro* in the presence of only small volumes of medium. With this equipment it has been found possible to incubate tissue slices for as long as 10 hours without appreciable diminution in their rates of oxygen uptake. Such prolonged and steady maintenance of oxygen consumption permits the inference that the tissues remain viable 2 to 3 times longer than in the conventional Warburg technique. The physical condition of the slices remains excellent for at least 6 hours, and the integrity of the tissue is reflected by the adequate recovery of cytochrome *c* after incubation.

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THE METABOLISM OF THE ORGANIC ACIDS OF TOBACCO LEAVES

XI. EFFECT OF CULTURE OF EXCISED LEAVES IN SOLUTIONS OF GLYCOLATE AT pH 3 TO pH 6

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Glycolic acid has assumed considerable importance in recent years as a component of plant and animal tissues. It was probably first encountered in nature in unripe grapes by Erlenmeyer in 1866 (1), and was identified in the juice of the sugar beet by von Lippmann in 1891 (2). Its presence in the juice of the sugar cane was thoroughly established by Shorey (3) in 1899. Glycolic acid is today recognized as one of the early products of photosynthesis (4–7), and an enzyme system capable of oxidizing it was detected some years ago in etiolated barley seedlings by Kolesnikov (8).

The nature of this enzyme system has been extensively studied by a group of workers in Burris' laboratory at the University of Wisconsin (9–12) who have found that the main product of oxidation is glyoxylic acid, that the enzyme is widely distributed in plants, and, specifically, that it is present in tobacco leaves. Kenten and Mann (13) have also studied a preparation of this enzyme obtained from tobacco leaves, and have suggested that oxalic acid may arise as a product of the further enzymatic oxidation of glyoxylic acid. Finally, Zelitch and Ochoa (14) have isolated the enzyme glycolic acid oxidase in highly purified form from spinach leaves, and have shown it to be a flavoprotein. They also confirmed the observation of Tolbert, Clagett, and Burris (10) that the primary product of oxidation is glyoxylic acid and found, as had Kenten and Mann, that the hydrogen peroxide produced reacts non-enzymatically with glyoxylic acid to give formic acid.

That glycolic acid may also be directly concerned in the metabolism of nitrogen compounds has been shown by Schou *et al.* (5), who found that glycolic acid labeled with C^{14} in either carbon atom, when administered to *Scenedesmus*, gives rise to labeled glycine and serine as major products. Tolbert and Cohan (15) have made similar observations with excised leaves of wheat and barley as well as with saps expressed from these tissues.

A metabolic relationship between glycolic acid and citric acid is evident from the observations of Zbinovsky and Burris (16), who infiltrated labeled glycolic acid into tobacco leaves and found that the specific activity of suc-

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cinic, oxalic, and malic acids was increased to approximately the same extent in light, although that of citric acid was only moderately affected. On the other hand, when the infiltrated leaves were held in darkness, the specific activity of citric acid was greatly increased and that of oxalic acid moderately; malic acid was somewhat less extensively labeled.

The obviously far-reaching relationships of glycolic acid in the metabolism of the organic acids of plant tissues suggested that a study of the effect of administering glycolic acid to tobacco leaves by the excised leaf culture technique might provide information upon the nature and extent of the reactions into which this substance may enter in the leaf. The outcome has been a demonstration that the formation of citric acid is enormously stimulated in darkness and that of oxalic acid is moderately stimulated. When glycolate is supplied at pH 5 and 6, malic acid increases slightly during the first 24 hours but diminishes subsequently. There was evidence that succinic acid as well as organic acids present in small amounts in the so called "undetermined" fraction also increase to some extent.

EXPERIMENTAL

The plants of *Nicotiana tabacum*, variety Connecticut shade-grown, were raised in the greenhouse on soil with occasional additions of nutrient solution to insure an adequate supply of nitrogen. The samples of leaves were collected by the statistical method (17) 53 days after the plants had been set out, ten samples of twenty leaves each being taken from twenty plants on July 6, 1953, ten fully developed leaves from each plant being used. The coefficient of variation of the fresh weight of the samples was 2.1 per cent and that of the total nitrogen content was 1.4 per cent; the samples were thus identical in initial composition within satisfactory limits.

The four culture solutions were 0.2 M with respect to glycolic acid and were adjusted with potassium hydroxide to pH 3, 4, 5, and 6; four samples were separately cultured in these solutions for 24 hours and four for 48 hours. In addition, one sample was cultured in water for 48 hours and one was dried at once for the zero time control. The dark room in which the leaves were treated was maintained at 24° and 75 per cent relative humidity.

The preparation of the samples for analysis and the analytical methods employed have been described in previous papers (18, 19). Glycolic acid was determined by the colorimetric procedure of Calkins (20) in appropriately pooled and concentrated fractions from a Dowex 1 column in the acetate form. D-Glyceric acid, which is present in these fractions in small amount, does not interfere significantly with the colorimetric procedure.

The control sample cultured in water increased nearly 13 per cent in fresh weight, but the leaves cultured in glycolate suffered some physiological damage. Most of them had small necrotic areas at the base where

they were immersed in the culture solution, and these areas frequently extended up along the main veins; a few leaves had necrotic areas extending into the lamina and some were mottled and yellow. Although most of the leaves were flaccid, the losses of fresh weight (Table I, Line 1) were much smaller than are observed when similar leaves are cultured in oxalic, acetic, or maleic acid, for, with these substrates, losses of the order of 50 per cent of the fresh weight occur in 48 hours. The average loss of fresh weight after 24 hours of culture in glycolate was 13 per cent, and after 48 hours it was 16.6 per cent. In spite of the evidence of damage, much of which occurred during the second 24 hour period of the culture, the main biochemical effects observed are clearly the result of the advent of glycolic acid and represent the response of the enzyme systems of the cells to the presence of this substance in unusual concentration.

In considering the analytical data in Table I, allowance should be made for a sampling error of the order of 2 per cent, and to this must be added an analytical error that, with such components as glycolic and succinic acids, may be as great as 10 per cent of the quantity measured, although with most components it is much less. Line 2 shows that the uptake of potassium from the culture solution at pH 3 was below the limit of detection but that the ash increased with the pH of the solution and with time. However, the uptake of solute was appreciably greater in the first 24 hours than in the second. The change in organic solids (*i.e.*, total solids minus ash) in Line 3 has been corrected in Line 4 by adding the weight of a quantity of carbon dioxide calculated from the increase in the alkalinity of the ash. It is assumed that this represents carbonate in the ash derived from the combustion of the organic acids and properly belongs with the organic solids. Because of the promotion of the respiratory loss of organic solids in leaves cultured in solutions of salts, most of the samples suffered a net loss in spite of the uptake of substantial amounts of organic acid.

Line 5 shows the increases in the alkalinity of the ash and Line 6 the effects upon the pH of extracts of the dried tissue. The uptakes of organic acid in Line 7 are calculated from the increases in the alkalinity of the ash by dividing by the respective fractions of glycolic acid which are neutralized at each pH, namely 0.13 at pH 3, 0.60 at pH 4, 0.94 at pH 5, and 0.99 at pH 6. These fractions were calculated from the dissociation constant ($pK' = 3.83$) of glycolic acid. The results for uptake at pH 3 obtained in this way are obviously meaningless since the change in the alkalinity of the ash was no greater than the experimental error. Accordingly, the quantities of glycolic acid actually found in these two samples are entered in Table I but are parenthesized to indicate that they are minimal estimates. The uptakes from the more completely neutralized solutions are proportional to the respective increases in the ash when the observa-

TABLE I

Effect of Culture in 0.2 M Solutions of Glycolate upon Composition of Excised Tobacco Leaves

The data represent gm. or milliequivalents per kilo of initial fresh weight of tissue.

Line No.		Control before culture	Changes during culture in darkness							
			Water	Potassium glycolate						
				pH 3		pH 4		pH 5		pH 6	
			48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.
1	Change in fresh weight, %	0	+12.6	−11.8	−16.8	−12.2	−15.8	−16.8	−21.1	−11.6	−16.6
2	Inorganic solids, *gm.*	20.0	−0.4	−0.4	−0.7	+4.6	+5.6	+8.2	+12.3	+8.2	+13.6
3	Organic solids, *gm.*	96.2	−7.7	−5.3	−10.7	−3.8	−7.0	−0.9	−1.5	−2.1	−4.6
4	" " corrected for CO_2 of ash, *gm.*	103.6	−7.8	−5.3	−10.9	−2.6	−5.3	+1.7	+2.5	+0.4	−0.4
5	Alkalinity of ash, *m.eq.*	334	−6.6	+1.0	−10.6	+55.9	+76.6	+117	+180	+115	+191
6	pH of extract of dry tissue	5.1	+0.1	−0.27	−0.32	+0.01	−0.03	+0.30	+0.31	+0.30	+0.34
7	Uptake of acid, *m.eq.*			(32)	(34)	93	128	125	193	116	193
8	" " " *gm.*			(2.4)	(2.6)	7.1	9.7	9.5	14.6	8.8	14.6
9	Total organic acids, *m.eq.*	314	−15	+19	+20	+57	+92	+96	+142	+83	+170
10	Malic acid, *m.eq.*	211	−45	−33	−68	−13	−51	+13	−20	+14	−25
11	Citric " "	35.9	+30.9	+8.7	+50.4	+29.8	+82.8	+30.3	+89.5	+24.8	+125
12	Oxalic " "	30.8	+1.1	−4.0	−2.0	+0.2	+0.2	+15.5	+23.4	+11.4	+16.2
13	Succinic acid, *m.eq.*	1	0.0	+4.0	+3.0	0.0	+7.7	+9.4	+10.7	+10.6	+10.6
14	Glycolic " "	2.3	+0.2	+32	+34	+29	+39	+14	+26	+15	+19
15	Unknown acid A, *m.eq.*	20	−5	+1	−2.2	+2.6	−0.1	−0.1	−5.3	−4.4	+3.5
16	Undetermined acid, *m.eq.*	14	+3	+10	+7	+8	+14	+13	+18	+12	+20
17	Protein N, *gm.*	2.72	−0.30	−0.31	−0.72	−0.16	−0.69	−0.03	−0.25	−0.10	−0.56
18	Starch, *gm.*	3.93	−3.07	−2.43	−3.07	−2.73	−3.00	−1.03	−1.68	−1.50	−2.49

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tions at 24 hours are compared with those at 48 hours. The data thus conform closely with the assumption that the increases in ash represent increases in potassium carbonate derived from the combustion of potassium glycolate and its metabolic products.

Line 9 shows the changes of total organic acids. The quantities are the sums of the titrations of the entire effluent from the analytical Dowex 1 column together with the separately determined oxalic acid. A loss of organic acids of the order of 15 m.eq. during culture of the leaves for 48 hours in water is commonly encountered, but the increases of about 20 m.eq. in the samples cultured at pH 3 indicate that some uptake of glycolic acid from this solution must have occurred. The larger increases in the leaves cultured at higher pH conform with what would be expected from the data for ash.

Malic acid (Line 10) decreased and citric acid (Line 11) increased in the sample cultured in water in the manner to be expected from earlier experiments, and there was a moderate stimulation of the respective changes in the amounts of these two substances in the leaves cultured for 48 hours in glycolate at pH 3. At pH 4, however, there is evidence of a diminished utilization of malic acid coupled with a marked stimulation of the formation of citric acid, and this effect is even more striking at pH 5 and 6. Under these last conditions, malic acid *increased* moderately for the first 24 hours and then diminished but, at the same time, the formation of citric acid was maintained at an accelerating rate. The outcome was that the accumulation of citric acid in 48 hours at pH 4, 5, and 6 was of the same order as has been observed when tobacco leaves are cultured in 0.2 M citrate at these same levels of acidity (21). The presence of a highly active citric acid-synthesizing mechanism is thus clearly indicated.

There was no evidence of an effect upon the content of oxalic acid (Line 12) when the leaves were cultured in water or in glycolate at pH 3 or 4, but at pH 5 and 6 oxalic acid increased strikingly. This is the first instance in the experience of this laboratory in which culture of tobacco leaves in an organic acid other than oxalic acid has led to a marked increase in the concentration of oxalic acid in the leaves.

Succinic acid is normally present in tobacco leaves in such small amounts as to be barely detectable by the titration of the appropriate fractions of the effluent from the Dowex 1 column. In Line 13, the quantity 1 m.eq. shown in the control sample represents an estimate for which the word "trace" might well be substituted; whether or not there was an increase during culture in glycolate at pH 3 is problematical, although this seems likely. At pH 4 and above, however, there was definite evidence of the accumulation of succinic acid in amounts approximately one-third of those found when similar leaves are cultured in succinate (22) at the same pH.

TABLE II

Metabolism of Glycolic Acid by Systems Present in Tobacco Leaves

Data expressed in terms of 1 kilo of initial fresh weight of leaves. For explanations of the derivation of these quantities, see the text.

Line No.		Changes during culture in darkness							
		Water	Potassium glycolate						
			pH 3	pH 3	pH 4	pH 4	pH 5	pH 5	pH 6	pH 6
1	Duration of culture, *hrs.*	48	24	48	24	48	24	48	24	48
2	Respiration loss, *gm.*	7.8	(7.6)	(13.5)	9.7	15.0	7.8	12.0	8.4	15.1
3	Glycolic acid metabolized, *m.eq.*		(0)	(0)	64	89	111	167	101	173
4	" " " as % uptake		(0)	(0)	69	70	89	86	87	90
5	Δ malic : Δ citric acids, *molar ratio*	2.2	5.7	2.0	0.6	0.9	0.7	0.3	0.8	0.3
6	Loss of organic acids, *m.eq.*				36	36	29	51	33	22
7	Glycolic acid converted to other acids, *m.eq.*				28	53	82	116	68	151
8	Δ succinic + Δ oxalic + Δ undetermined acids, *m.eq.*				8	22	38	52	34	47
9	Glycolic acid available for conversion to citric acid, *m.eq.*				19	32	44	64	34	104
10	Δ malic acid, *mmoles*				−6.3	−25.4	+6.6	−9.8	+6.8	−12.3
11	Citric acid assumed to be derived from malic acid, *mmoles*				3.13	12.7		4.9		6.2
12	Δ citric acid, *mmoles*				+9.93	+27.6	+10.1	+29.8	+8.27	+41.8
13	Citric acid from source other than malic acid, *mmoles*				6.8	14.9	10.1	24.9	8.27	35.6
14	Ratio, Line 9 to Line 13				2.8	2.1	4.3	2.6	4.1	2.9
15	" " 9 " " 3				0.30	0.36	0.40	0.38	0.34	0.60

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Glycolic acid (Line 14) is also normally present in tobacco leaves in only small amounts. It is not detectably altered during culture of the leaves in water, but an increase of about 34 m.eq. was found in the leaves cultured in glycolate at pH 3. Although it seems unlikely that this quantity furnishes an accurate measure of the glycolic acid that was taken up, there was no clear evidence of metabolic effects which could be specifically attributed to the presence of this substance. The moderate stimulation of the formation of citric acid that occurred may merely have been an expression of the increased rate of the general metabolism brought about by culture of the leaves in the presence of a salt. Glycolic acid accumulated at pH 4 to only approximately the same extent as at pH 3 in spite of the large uptakes shown in Line 7, and above pH 4 even less was found. This substance must, therefore, readily enter into the organic acid metabolism of the tissue.

The component designated "Unknown acid A" (Line 15), which is eluted from the Dowex 1 column along with citric acid, is now known to be a mixture which contains trace amounts of malonic and isocitric acids together with a major component as yet unidentified. No increase in isocitric acid was detected and, considered as a whole, this mixture underwent only small and irregular changes as a result of culture of the leaves in glycolate; its metabolic significance thus awaits more detailed study. The group of components designated "Undetermined acid" (Line 16) represents those substances (with the exception of succinic and glycolic acids) which are eluted in advance of malic acid. D-Glyceric, quinic, and aspartic acids have been recognized in small amounts in this mixture, and there are several other acids present. As a whole, this group of substances was markedly increased by culture of the leaves in glycolate. Glyoxylic acid, if present, would be included in this group. However, it is doubtful whether glyoxylic acid is sufficiently stable to survive the drying of the leaves and subsequent evaporation of the formic acid eluate from the Dowex 1 column. At all events, none was detected in any of the present samples.

The protein nitrogen (Line 17) diminished during culture for 48 hours in water by 11 per cent of the amount present in the control. This is a somewhat greater loss than is usually observed, but, even so, the extent of proteolysis was approximately doubled in the leaves cultured for 48 hours in glycolate at pH 3, 4, and 6. No reason can at present be assigned for the relatively smaller loss of protein nitrogen from the samples cultured at pH 5. However, a somewhat lower level of total metabolism in these two samples is also suggested by the data for the loss of starch (Line 18). Nearly three-quarters of the starch disappeared from the other samples cultured either in water or glycolate for 48 hours.

In Table II are collected data derived from the analytical results in Table

I. The respiration losses in Line 2 are the algebraic sums of the changes in corrected organic solids and the estimated uptakes of glycolic acid for each sample. At pH 3, it has been necessary to use the data for the quantities of glycolic acid found by analysis in the samples since no other estimate of the uptake was available. Accordingly, the respiration loss in these two instances is underestimated by such amounts of glycolic acid as may have undergone metabolic reactions during the culture period. Reasonable magnitudes are nevertheless obtained, and it would thus appear that the acquisition of solute from the culture solution at this low pH required the expenditure of respiratory energy in amounts comparable with those observed at the higher pH of the other solutions. The value 7.8 gm. for the loss of organic solids during culture in water is somewhat higher than is commonly observed, and suggests that this lot of leaves was characterized by an unusually vigorous metabolism, a conclusion that may also be drawn from the losses of protein nitrogen and starch shown in Table I. No definite explanation can be advanced for the somewhat lower level of respiratory loss from the two samples cultured at pH 5, but the effect correlates with the completely independent data for the smaller losses of both protein and starch from these samples in comparison with those at lower and higher pH.

Line 3 of Table II shows the estimated quantities of glycolic acid which were metabolized by the tissues during the culture period. The data are the differences between the respective uptakes and the amounts of glycolic acid found by analysis. It is obvious that the rate of metabolism of glycolic acid in the first 24 hours of culture was somewhat greater than that in the second 24 hour period. However, Line 4 shows that the relative proportion of the acquired glycolic acid which was metabolized at each pH was essentially constant throughout the 48 hour period. Furthermore, the extent to which glycolic acid was converted to other substances at pH 5 and 6 is comparable to that observed for succinic acid under similar conditions (23). Glycolic acid must therefore be rated, along with succinic acid, as an extremely active metabolite in tobacco leaf tissue.

It is unfortunate that no data are available from which the extent to which glycolic acid is metabolized at pH 3 may be calculated. The trend of the data at the higher pH values would suggest that the proportion may be fairly small, but that *none* of the glycolic acid taken up was converted to other substances seems improbable; the zeros in Lines 3 and 4 of Table II are accordingly parenthesized to indicate doubt.

The molar ratio of the loss of malic acid to the increase of citric acid in each of the samples is shown in Line 5. As has been repeatedly observed in tobacco leaves cultured for 48 hours in water, this molar ratio is close to 2 (24), and the metabolism of malic acid in this particular set of samples

may therefore be assumed to have been normal. When cultured at pH 3 in glycolate, however, the ratio of the loss of malic acid to the gain of citric acid during the first 24 hours was 5.7. In the second 24 hour period, it dropped to 1.3 and for the entire 48 hour period was 2.0. Accordingly, if one assumes that the citric acid produced in 48 hours in the culture at pH 3 was derived entirely from malic acid, it follows that a considerable part of the malic acid must have been converted into substances which, for a time, remained as intermediate products. However, it is by no means certain that glycolic acid had no share in the synthesis of citric acid at pH 3; all that seems certain is that enough malic acid disappeared in 48 hours to account for the increase in citric acid observed.

The molar ratios of malic acid decrease to citric acid increase in the less acid culture solutions are all smaller than unity, and at pH 5 and 6 drop to 0.3 in the 48 hour cultures. Obviously, much of the citric acid must have arisen from some source other than malic acid, and suspicion naturally falls upon the glycolic acid which was extensively metabolized in these samples. Comparison of the data for the losses of glycolic acid in Line 3 of Table II with those for the increases of citric acid in Line 11 of Table I shows that, in terms of milliequivalents, from 2 to 4 times as much glycolic acid disappeared in 24 hours as there was citric acid produced, whereas, over the 48 hour period, the ratios ranged from 1.1 to 1.8; accordingly there would appear to have been an adequate supply of glycolic acid to account for the increase in citric acid.

A more detailed examination of this possibility is shown by the calculations in Lines 6 to 14 of Table II. The differences between the uptakes of glycolic acid and the increases in total organic acids in Line 6 may be assumed to represent the amounts of organic acid that disappeared entirely as the result of respiratory or other processes which removed titratable acidity during the culture period. If this loss is assumed to have fallen exclusively upon the acquired glycolic acid,[1] the differences between Lines 3 and 6 then show the amounts of glycolic acid which were converted into other stable organic acids. Line 8 gives the sums of the increases in succinic, oxalic, and undetermined acids from Table I, and, to take the most unfavorable possible case, it is assumed that these increases arose exclusively from the metabolism of glycolic acid. Line 9, the differences between Lines 7 and 8, then shows the amounts of glycolic acid which, on these assumptions, were available for conversion into citric acid. However, another possible source of citric acid exists; namely, that part of the malic

[1] Conversion of glycolic acid to glycine and serine, as has been observed to occur in wheat and barley leaves by Tolbert and Cohan (15), could account for some of the loss of titratable acidity. Any glycolic acid converted to glyoxylic acid or formic acid would also escape detection.

acid which disappeared during the culture period. On the assumption that the molar ratio between loss of malic and gain of citric acid, found valid for the water control and for the culture in glycolic acid at pH 3 for 48 hours, is also applicable in the cultures at higher pH values, Line 11 gives, in millimoles, the amounts of citric acid which may have arisen from malic acid. Line 12 shows the observed increases in citric acid, also expressed in millimoles, and Line 13 the differences between Lines 11 and 12. These are, accordingly, the amounts of citric acid which may have been derived from glycolic acid. Line 14 gives the molar ratios between the amounts of glycolic acid which may have been converted into citric acid (Line 9) and the amounts of citric acid that may be supposed to have arisen from this source.

The mean of the six calculations is 3.1 ± 0.9; accordingly, when all of the other likely fates of glycolic acid are taken into consideration, there still remains a sufficient supply, as measured by titratable acidity, to account for the observed extensive formation of citric acid. In view of the numerous assumptions that have been made, no stress can be placed upon the apparently stoichiometric ratio obtained, for the uncertainty is of the order of 30 per cent. Nevertheless, when sufficient knowledge of the enzyme systems involved has been accumulated and precise mechanisms can be suggested, provision must be made for the possibility of an extremely efficient conversion of glycolic acid to citric acid.

Line 15 of Table II shows the fractions of the total quantity of glycolic acid metabolized (Line 3) which were apparently utilized for the formation of citric acid. The first five figures agree closely with each other and suggest that, on the average, a little more than one-third of the available glycolic acid was so used. However, for reasons that are not apparent, the formation of citric acid was greatly stimulated in the second 24 hour period of the culture at pH 6, and the fraction metabolized to citric acid over the entire 48 hour period accordingly rose to 60 per cent. The desirability of the examination of leaves cultured in glycolate at pH 7 is obvious.

DISCUSSION

The present results provide confirmation of the observations of Zbinovsky and Burris (16) on the behavior of glycolic acid labeled with C^{14} when administered to tobacco leaves. The high specific activity they found in the citric acid of leaves held in darkness after infiltration with glycolic acid correlates well with the greatly promoted synthesis of this substance under the conditions of the present experiments, and provides convincing evidence that enzymatic mechanisms exist whereby glycolic acid is readily converted to citric acid. Their observation that malic acid acquires only a little activity in darkness correlates with the observation that malic acid is normally a substrate of reactions that lead to the synthesis of citric acid. The

drain upon malic acid at pH 3 and 4 of the culture solution in the present instance was sufficient to prevent any accumulation of this substance; at pH 5 and 6, however, accumulation of a small but significant amount occurred during the first 24 hours. Later, however, malic acid diminished again. It would appear that glycolic acid can be transformed into a substance which is common to the mechanisms whereby both malic and citric acids are produced. Furthermore, the small magnitude of the changes in the amounts of malic acid which occurred when glycolic acid was made available indicates that this substance exerts a "sparing" action on the utilization of malic acid for the synthesis of citric acid analogous to that observed when fumaric acid is supplied (25).

A somewhat different interpretation of the behavior of malic acid in these samples should not, however, be overlooked. It has been shown that the culture of tobacco leaves in citric acid at pH 5 and 6 gives rise to an increase of malic acid (21). Thus, in a situation such as the present, in which the formation of citric acid is notably promoted, there is a possibility that the temporary increase of malic acid observed at pH 5 and 6 may have arisen from glycolic acid in an indirect manner via citric acid rather than more directly.

The stimulation of the formation of oxalic acid at pH 5 and 6 of the culture solution would also be anticipated from the results of Zbinovsky and Burris, and provides confirmation of the suggestion of Kenten and Mann (13) that oxalic acid may arise from the further oxidation of the glyoxylic acid produced by glycolic acid oxidase. However, the conversion of glycolic acid to oxalic acid under the conditions of the present experiments was considerably less extensive than the conversion to citric acid. The increase of oxalic acid represented only 14 per cent of the glycolic acid metabolized (Table II, Line 3) in 24 and in 48 hours at pH 5, and only 11 and 9 per cent in the same time intervals at pH 6. Within the limits of error, these are constant fractions of that part of the glycolic acid which underwent chemical change over this pH range. Nevertheless, the demonstration that such a conversion may occur throws far more light than has hitherto been available upon the origin and position of oxalic acid in the general metabolism. Taken together with earlier observations of the stability of oxalic acid administered to the leaves (22), as well as with the results of Zbinovsky and Burris, who noted almost negligible effects when labeled oxalic acid was supplied to tobacco leaves, this substance appears to originate from glycolic acid and is definitely an end-product of the organic acid metabolism.

One further point merits attention; namely, the apparently lower level of total metabolism in the leaves cultured at pH 5 as shown by the loss of organic solids. The pH of this culture solution is so close to the acidity

of extracts of the control sample that the acquisition of a substantial quantity of buffered solution should present no problem with respect to the maintenance of a suitable pH reaction of the cell contents. Thus, one might expect to find evidence of diminished metabolic stress during culture at pH 5. On the other hand, when equally large quantities of glycolic acid buffered at pH 6 are taken up, the cells might be assumed to respond to the excess of alkali with the promotion of reactions such that the loss of carboxyl groups is minimized. In the sample cultured for 48 hours at pH 6, the loss of titratable acidity (Table II, Line 6) from the system was less than one-half of that observed at pH 5, and the synthesis of citric acid from glycolic acid by reactions that apparently involved no loss of titratable acidity was greatly promoted. Such reactions may well require the expenditure of additional respiratory energy and this is shown to have occurred (Table II, Line 2).

At pH 4 or below, however, the problem is to deal with an excess of acidity. The response of the leaves was apparently to minimize the uptake of glycolic acid (Table I, Line 7). There was no strikingly increased loss of organic acidity at pH 4 by what may have been decarboxylation reactions (Table II, Line 6), as has been observed to occur when malic acid is administered to the leaves under similar circumstances (24), but the extracts of the samples cultured at pH 3 became distinctly more acid (Table I, Line 6), and the expenditure of respiratory energy appears to have been increased. Nevertheless, attractive as this hypothesis may be, it fails to account for the minimized hydrolysis of the protein at pH 5, a response that appears to be unique for the case of tobacco leaves cultured in glycolic acid.

At the present time, it is futile to discuss possible enzymatic mechanisms in the leaf cells whereby such transformations as have been observed may be brought about. Knowledge of the organic acid composition of the leaves is still notably incomplete, since techniques have not yet been developed for the examination of fresh tissue so that changes of volatile and of unstable acid components can be measured. Such components may well occupy key positions in the actual reactions which occur in the cells.

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SUMMARY

Excised tobacco leaves (*Nicotiana tabacum*, var. Connecticut shade-grown), when cultured in 0.2 M solutions of potassium glycolate, take up

only small amounts of glycolic acid at pH 3 but increasingly large amounts at pH 4 and 5. The uptake at pH 6 is approximately the same as at pH 5. At pH 4, 70 per cent of the glycolic acid acquired in 48 hours is rapidly metabolized and, at pH 5 and 6, this figure rises to nearly 90 per cent. Glycolic acid thus ranks with succinic acid as an extremely active metabolite in the tobacco leaf. The main product of the metabolism is citric acid, and the stimulation of the formation of citric acid is such that this substance accumulates in amounts comparable with those found when citric acid is itself supplied under the same conditions. However, a moderate and nearly constant fraction of the acquired acid is oxidized to oxalic acid, and there is a small increase in succinic acid as well as in a group of acids, present in small proportions, the identity of which is still incompletely known. Glycolic acid is thus apparently capable of being metabolized in the tobacco leaf by mechanisms which lead to the accumulation, in addition to citric acid, of numerous products in small amounts. Although some of these substances may ultimately be found to fit into a general metabolic scheme of which citric acid is the final and major product, the formation of oxalic acid appears to represent a side reaction.

With the exception of oxalic acid itself, glycolic acid is the only organic acid hitherto studied by the excised leaf culture technique which contributes to the accumulation of oxalic acid in tobacco leaves. Glycolic acid is, therefore, a possible source of the substantial amounts of oxalic acid normally found in this species.

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URINARY EXCRETION OF METHYLMALONIC, α,α-DIMETHYLSUCCINIC, AND OTHER ETHER-SOLUBLE ACIDS IN RATS*

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Dietary liver necrosis in rats can be prevented or modified by the addition to the diet of cystine or vitamin E or of several antibiotics, in particular Aureomycin or penicillin (1). Metabolic products of rats fed the basal diet which produces necrosis are modified by the addition of these supplements.

A variation in the urinary excretion of certain ether-soluble acids was noted (2). Two of these acids were previously identified (2) as methylmalonic acid, which had been reported in rat urine, and α,α-dimethylsuccinic acid, not previously found in biological fluids.

Further investigations have been made of the urinary excretion of these ether-soluble acids in rats fed similar diets and similar supplements.

Method

The methods used are essentially the same as those previously described. Male weanling rats (Sprague-Dawley) weighing approximately 45 gm. were kept in individual cages and given the basal necrogenic diet[1] with a daily vitamin supplement. Aureomycin-HCl (Lederle) 25 mg., penicillin (*N*,*N*′-dibenzylethylenediamine, Wyeth) 5 mg., cystine 25 mg., or vitamin E 0.5 mg. was added to the daily rations of the treated groups of animals. In addition, one control group of rats was fed a complete non-necrosis-producing diet, laboratory chow (Purina).

Urine, collected from each rat under toluene, was acidified, and extracted with ether continuously for 3 hours (3, 4). The ether was evaporated and an aliquot of the residue was placed on Whatman No. 1 chromatogram paper. Chromatograms were developed with butanol, acetic acid, and water (4:1:1), dried, examined under fluorescent light, and sprayed with brom cresol green for identification of the acids. 5 μmoles of the acid ex-

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[1] The basal diet consists of corn-starch 79, bakers' yeast 18 ("vitamin B_1 yeast," Distiller's Corporation, Ltd., Glasgow), salt mix No. 2, U. S. P. 3, and peanut oil 0.5. Approximately 8 gm. were fed daily.

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tract were placed on similar paper and developed with the same reagents. Without having been sprayed, 1 cm. squares were cut from the paper with controls above and below the desired R_F and eluted with ether, which was evaporated. The residue was dissolved in 1 ml. of water and titrated with 0.001 N NaOH, and the relative concentrations of acids at each R_F were determined.

For preparation of the individual acids, large quantities of rat urine were extracted with ether, and the dried extract was placed on a 9 mm. × 150 mm. Dowex 1 × 10 (Cl^-) column and eluted in 12 ml. fractions with increasing concentrations of hydrochloric acid at 25°. Almost all the acids were eluted in 15 liters of wash. Final concentration of hydrochloric acid was 0.02 N. Succinic acid was isolated by using a similar Dowex 1 × 10 column in formate form and eluting with 700 ml. of formic acid of increasing strength to 0.6 N. Fractions from this column were 3 ml.

TABLE I

Daily Urinary Excretion of Ether-Soluble Acids (ESA) by Rats on Necrogenic Diet for 50 Days

Supplement to basal diet	No. of rats tested	ESA	$\frac{\text{ESA}}{\text{Creatinine}} \times 10$
		μmoles	
None	10	440 ± 20.6	153 ± 11.3
Vitamin E	10	665 ± 28.4	155 ± 12.4
Aureomycin	10	119 ± 11.0	47 ± 8.2
Penicillin	4	101 ± 7.5	67 ± 4.5
Cystine	5	136 ± 14.2	86 ± 10.6
Chow (complete diet)	4	156 ± 19.8	59 ± 8.7

The elutions were lyophilized, and the solids were taken up in small amounts of water, from which the acids were crystallized, and identified by elemental analysis, mixed melting point, and paper chromatography.

Total ether-soluble acids of the urine were titrated with 0.1 N NaOH. Other chemical determinations were previously described (2).

Results

Ether-soluble acid excretion of these groups of rats was similar to that previously described, typical daily ether-soluble acid excretion results being presented in Table I. It is notable that the animals that were fed a complete diet (chow) and those fed the necrosis-producing diet supplemented with Aureomycin, penicillin, or cystine had significantly lower ether-soluble acid excretion than the rats fed the necrosis-producing "basal" diet alone or the "basal" diet supplemented with vitamin E.

Following paper chromatography of the ether-soluble acids, four acidic

spots were identified (Table II). All of the acids seen on the chromatogram have now been identified.

The acid spot of R_F 0.95 is due to α,α-dimethylsuccinic acid. The rats on a "basal" diet or on a diet supplemented with vitamin E excrete proportionally little of this acid. The rats on the chow diet, or on a "basal" diet supplemented with Aureomycin, penicillin, or cystine, excrete approximately 50 per cent of their ether-soluble acids in this form. Because the relative amounts of each acid were determined after eluting from chromatogram paper, it cannot be stated with certainty that the absolute amount of α,α-dimethylsuccinic acid is different in the animals that were fed the six different supplements.

TABLE II

Paper Chromatogram of Ether-Soluble Acids (Per Cent)*

Supplement to basal diet	R_F			
	0.45	0.80	0.86	0.95
None	5	5	75–80 (76)	10–15 (14)
Vitamin E	5	5	75–80 (78)	10–15 (12)
Aureomycin	10	25–30 (26)	15–40 (32)	30–60 (32)
Penicillin	10	25–30 (28)	15–40 (24)	20–50 (38)
Cystine	10	25–40 (26)	15–40 (21)	10–50 (43)
Chow	10	25–40 (27)	15–40 (26)	10–50 (37)

* Extracts were placed on Whatman No. 1 chromatogram paper and developed with butanol, acetic acid, and water (4:1:1). 1 cm. squares of paper were cut in the areas of the R_F stated, eluted with ether, and titrated with 0.001 N NaOH. The values represent the range of eight determinations, those in parentheses being the means of these determinations.

The spot of R_F 0.86 is composed of two acids: a non-fluorescent acid which has been identified as methylmalonic acid and a fluorescent acid which is hippuric acid. The hippuric acid excretion of all groups is approximately equal (2). The methylmalonic acid excretion, however, accounts for approximately 70 per cent of the total ether-soluble acid excretion of the rats on the "basal" diet and of the rats on the vitamin E-supplemented diet, but only approximately 10 per cent of the total ether-soluble acid excretion of the rats on the chow diet, or of the rats on the "basal" diet supplemented with Aureomycin, penicillin, or cystine.

The spot of R_F 0.80 was found to be due to two acids: succinic acid in the Aureomycin, penicillin, and cystine-supplemented groups of rats and a mixture of succinic acid and *cis*-aconitic acid in the rats that were fed the chow. There is almost no spot of R_F 0.80 in the rats on the "basal" diet or the "basal" diet supplemented with vitamin E.

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Spot R_F 0.45, which was previously reported, has been found to be due to the technique of extraction and not to a constituent of urine.

In summary, rats that were fed the "basal" diet or the "basal" diet supplemented with vitamin E excrete relatively large quantities of ether-soluble acid, most of which is methylmalonic acid. Rats fed the "basal" diet supplemented with Aureomycin, penicillin, or cystine or rats fed a complete diet excrete relatively smaller amounts of ether-soluble acids. These acids consist largely of succinic and α,α-dimethylsuccinic acids and of little methylmalonic acid.

Since animals on the "basal" diet supplemented with vitamin E usually excrete large quantities of ether-soluble acids, while those on diets supplemented with Aureomycin always excrete small quantities of ether-soluble acids, these supplements were fed simultaneously. One group of eight rats was fed the "basal" diet plus vitamin E for 60 days, and another group of eight rats simultaneously was pair-fed a "basal" diet plus Aureomycin. Then, four rats of the vitamin E-supplemented group had Aureomycin added to their rations for 21 days, and four animals of the group with Aureomycin had vitamin E added to their rations for 21 days. The other rats of each group remained on the original supplement.

TABLE III

Effects of Additions of Aureomycin to Vitamin E-Supplemented Animals and of Vitamin E to Aureomycin-Supplemented Animals on Daily Ether-Soluble Acid Excretion (ESA)

Diet supplement	Rat No.*	ESA		$\frac{\text{ESA}}{\text{Creatinine}} \times 10$	
		Average	Range	Average	Range
		μmoles	*μmoles*		
Vitamin E	651–654	514	190–890	215	89–350
" " + Aureomycin	655–659	236	140–308	92	72–110
Aureomycin	640–643	152	138–166	49	43–69
" + vitamin E	645–648	186	132–224	62	43–87

* Rats 651 to 659 were fed diets supplemented with vitamin E for 60 days. Rats 640 to 648 were simultaneously pair-fed similar diets, supplemented with Aureomycin. After the 60th day, Aureomycin was added to the vitamin E-supplemented diet of Rats 655 to 659, and vitamin E was added to the Aureomycin-supplemented diet of Rats 645 to 648 for 21 days (diet Day 61 to 81). Ether-soluble acid excretion was measured simultaneously in the sixteen rats on Days 74 to 81.

The results (Table III) indicate that the high ether-soluble acid excretion of rats fed vitamin E can be reversed to a low level when Aureomycin is added to the ration. In contrast, the low urinary excretion of ether-soluble acids by rats on an Aureomycin-supplemented diet is not affected by the addition of vitamin E to the ration.

Fig. 1 presents a graph of the data of rats on a "basal" diet intermittently supplemented with Aureomycin and penicillin. It is seen that Aureomycin depresses ether-soluble acid excretion and that acid excretion increases when

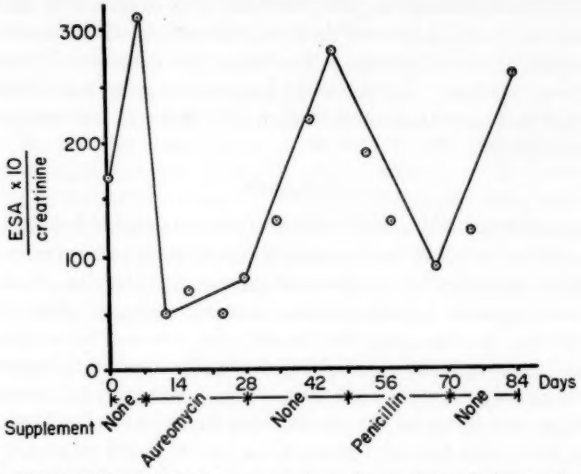

FIG. 1. Effect of intermittent Aureomycin and penicillin administration on ether-soluble acid excretion.

TABLE IV

Daily Ether-Soluble Acid Excretion of Rats on Stated Diets Supplemented with Ether-Soluble Acids (10 Mg. per Rat per Day)*

Basal diet supplement	Acid supplement									
	None	α,α-Dimethylsuccinic	None	*cis*-Aconitic	None	Citric	None	Succinic	None	Isosuccinic
None	262	157	214	231	150	159	165	206	175	222
Vitamin E	145	114	129	207	158	208	219	175	230	224
Aureomycin	82	67	38	72	40	51	45	46	35	45

* All the values are (ESA/creatinine) × 10, and are the averages of the results of six animals, a number too small to allow valid statistical analysis with the scatter observed. The acids were fed successively to the same animals. Each acid was fed for 2 weeks, followed by 2 weeks of the stated diet without acid supplement. Acid excretion was measured during the last 3 days of feeding of each supplement.

the animal is taken off Aureomycin and fed only the "basal" diet. Similarly, a supplement of penicillin decreases acid excretion, and such excretion again increases when penicillin is removed from the diet.

An attempt was made to determine whether any of these acids was toxic or a cause of liver disease. Pure preparations were obtained or synthe-

sized[2] and fed to the rats successively on the different diets, as recorded in Table IV. α,α-Dimethylsuccinic acid decreases slightly the total acid excretion in the animals on the "basal" and vitamin E-supplemented diets, and succinic acid decreases slightly the total acid excretion of the animals on the vitamin E-supplemented diet. *cis*-Aconitic acid increases acid excretion slightly in all the groups. No change was detectable in the clinical appearance of the rats. Furthermore, paper chromatograms following the acid feedings indicated that much of each acid that was fed was apparently excreted unchanged.

DISCUSSION

The high ether-soluble acid excretion of the rats on the basal necrogenic diet and on the vitamin E-supplemented diet appears to be abnormal, since the rats fed a chow diet have a low acid excretion. Likewise, the excretion of the individual acids by animals on a diet supplemented with vitamin E is similar to that of animals on the "basal" diet, whereas the acids excreted by rats on a diet supplemented with Aureomycin, penicillin, or cystine are similar to those of rats on the chow diet. Since vitamin E is the supplement which best protects a rat on the "basal" diet from developing liver necrosis, it appears that ether-soluble acid excretion *per se* is not related to dietary liver necrosis.

A high level of ether-soluble acid excretion and excretion of methylmalonic acid, therefore, must be a manifestation of a metabolic abnormality of rats fed a basal necrosis-producing diet. Furthermore, Aureomycin, penicillin, and cystine correct this abnormality, but supplements of vitamin E do not. As yet, it has not been determined whether the correction of this abnormality is owing to a supplementation of a dietary deficiency, an effect of antibiotics on the intestinal flora (6, 7), or a direct metabolic antibiotic effect. The mode of action of these substances can be determined, finally, only by a study of germ-free animals. At present, however, a metabolic effect of the antibiotics seems the most likely hypothesis because of the duration of the effect.

The long and persistent effect of Aureomycin and penicillin is unusual. Several systems (8, 9) have been examined for a long term antibiotic effect. For example, Huerga *et al.* (8) found that Aureomycin depresses trimethylamine excretion after oral administration of choline, but that this depression is spontaneously reversible after about 10 days. This escape effect was also noted in rats by Cunningham[3] in our laboratory. The depression

[2] α,α-Dimethylsuccinic acid was prepared by treating dimethyldihydroxyresorcinol with potassium permanganate (5). Methylmalonic acid was kindly supplied by Dr. H. Mayer Kaupp of Heidelberg, and the remainder of the acids are available commercially.

[3] Cunningham, Charles, personal communication.

of ether-soluble acid excretion for as long as a year in animals fed Aureomycin or penicillin, and the return to high values when these drugs are discontinued is the first long term effect of continuous administration of Aureomycin or penicillin that has been noted.

The particular ether-soluble acids found are unusual. Succinic acid has been reported in normal human and rat urine (10, 11) and is a normal fermentation product of yeast (12). It presumably is a normal metabolic product. Its absence from the urine of rats on the "basal" diet or the "basal" diet supplemented with vitamin E suggests a deficiency in the Krebs cycle in the latter animals. While the status of methylmalonic acid in the Krebs cycle is as yet undetermined, its close relationship to malonate also suggests involvement of the Krebs cycle. As none of the acids, at least in the doses fed, had any obvious effect on the metabolism or well being of the rats, these acids cannot be considered metabolic poisons of the Krebs cycle.

The source of methyl malonate is unknown. Methyl malonate was reported in normal rat urine and was found increased following anthracene poisoning (13). Recently, considerable interest in methylmalonic acid has developed as a result of the work of Coon (14) and others. Flavin (15) and Katz and Chaikoff (16) have reported the formation of methylmalonic acid from propionate and carbon dioxide, and Coon (14) has proposed that methyl malonate or its coenzyme A thiol ester may be a product of valine metabolism. As the yeast in the "basal" diet contains 5.3 per cent valine (17), this could well be a source of the dicarboxylic acid. It is of interest in this regard that several investigators (18, 19) have suggested that coenzyme A may be deficient in rats developing liver necrosis.

The source of α,α-dimethylsuccinic acid in rats is unknown. It is not known to occur naturally in biological fluids. Because α,α-dimethylsuccinic acid has been isolated as an oxidative product of vitamins A and B_{12}, *limited* experiments were performed with low vitamin A, high vitamin A, and high β-ionone supplements to the "basal" diet, without appreciable change in ether-soluble acid excretion. Vitamin B_{12} is an unlikely source of these acids, as there was no vitamin B_{12} in the diet.

cis-Aconitic acid was isolated only from the urine of rats fed a chow diet. It has previously been found in normal human urine (10). Because of finding it only in the animals on a normal diet, few studies have been carried on with this acid. The reason for its absence in the urine of rats fed the basal necrogenic diet with or without supplements remains obscure.

SUMMARY

1. Urine of rats fed a liver necrosis-producing diet, or this diet supplemented with vitamin E, contains the following ether-soluble acids: succinic,

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methylmalonic, α,α-dimethylsuccinic, and hippuric. Quantitatively, total ether-soluble acid excretion is high, chiefly owing to a large amount of methylmalonic acid.

2. Urine of rats fed the same diet supplemented with Aureomycin, penicillin, or cystine contains the same acids. Urine of rats fed a chow diet, in addition, contains *cis*-aconitic acid. Total ether-soluble acid excretion of all these groups of animals is low, and the predominant acid is α,α-dimethylsuccinic, the source of which is unknown.

3. None of these acids, when fed to the rats, was found to be toxic.

4. The best protection against dietary liver necrosis resulted from vitamin E supplementation. Aureomycin, penicillin, and cystine supplements were less consistently effective. No common factor in these five diets and supplements was found to explain protection against dietary necrosis.

5. The effect of Aureomycin or penicillin on ether-soluble acid excretion is prolonged. This effect was noted for a year on continuous administration, disappeared when the drug was withheld, and reappeared upon restarting its administration. No other effect *in vivo* of Aureomycin or penicillin has been noted to persist for this length of time.

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CONVERSION OF CINNAMALDEHYDE TO STYRENE BY A YEAST MUTANT

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During our investigation on the metabolism of biochemical mutants of yeast (*Saccharomyces cerevisiae*), we found that one mutant (strain 3212) produced an abnormal, unpleasant odor during fermentation in the presence of cinnamaldehyde. The compound associated with the obnoxious odor was isolated and identified as styrene. Since this unusual formation of a hydrocarbon during yeast fermentation has not been described previously in the literature, our observations are reported in this paper.

EXPERIMENTAL

Isolation and Identification of Styrene from Fermented Medium—A synthetic medium of the following composition was used as charge for yeast fermentation: sodium succinate 2.8 gm., succinic acid 7 gm., asparagine 20 gm., ammonium sulfate 5 gm., monobasic ammonium phosphate 6 gm., potassium chloride 4 gm., zinc sulfate 0.3 gm., ferrous ammonium sulfate 25 mg., manganese sulfate 20 mg., copper sulfate 20 mg., inositol 0.4 gm., biotin 400 γ, pyridoxine 25 mg., thiamine 25 mg., and calcium pantothenate 25 mg. These ingredients were dissolved in 4 liters of saturated aqueous solution of cinnamaldehyde and seeded with 520 gm. of strain 3212 press-cake (30 per cent solids). The carbon source provided was composed of 30 per cent sucrose in saturated cinnamaldehyde solution; it was fed continuously into the fermentation tank at a rate of 100 ml. per hour. Since a high concentration of cinnamaldehyde in the medium was known to inhibit yeast fermentation, it was avoided in this manner during the 12 to 14 hour fermentation.

Preliminary attempts to concentrate the harsh smelling compound by vacuum or steam distillation were unsuccessful. Isolation was finally accomplished by attaching a dry ice trap to the closed fermentor and flushing the medium with nitrogen during fermentation to remove the volatile substances. About 1 to 2 ml. of the compound was isolated from 5 liters of medium during a 12 hour fermentation.

The isolated compound was identified as styrene from its ultraviolet absorption spectrum, shown in Fig. 1, and from its physical constants as well as from the melting point of its brominated derivative (Table I).

Mechanism of Styrene Formation—The mechanism for the conversion of cinnamaldehyde into styrene by yeast fermentation is only partly understood. It was first thought that cinnamaldehyde might be oxidized to cinnamic acid which was then converted to styrene by simple decarboxylation as shown in the accompanying structure. To test this hypothesis, the formation of cinnamic acid was measured by the change in the ultra-

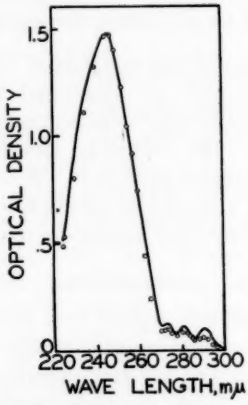


FIG. 1. Comparison of the ultraviolet absorption spectra of "unknown" (○) and styrene (solid line). Measurement made in absolute ethanol. The absorption maxima at 248 mμ in both curves were adjusted to the same height for comparison.

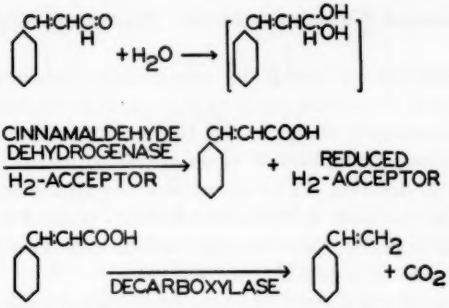
TABLE I

Physical Properties of Unknown Substance

The melting point of the dibromide derivative was 74–75° for "unknown" and styrene.

	M.p.	B.p.	Sp. gr.	Refractive index
	°C.	°C.		
"Unknown"	−33 to −35	140–141	0.888	1.540
Styrene	−35	145	0.906	1.547

violet absorption spectrum of the medium during the incubation of strain 3212 with cinnamaldehyde in sucrose-phosphate buffer medium. Fortunately, the absorption maxima of cinnamyl alcohol, cinnamic acid, and cinnamaldehyde differed widely enough for easy identification. The wavelengths of maximal absorption and the molar extinction coefficients of these compounds in aqueous medium are as follows: cinnamyl alcohol, 250 mμ, $E = 1.70 \times 10^4$; cinnamic acid, 271 mμ, $E = 2.97 \times 10^4$; cinnamaldehyde, 291 to 292 mμ, $E = 2.30 \times 10^4$.



All measurements were made in the following system: 2 ml. of a 10 per cent sucrose solution, 1 ml. of phosphate buffer (0.1 M, pH 6.0), 2 ml. of saturated solution of cinnamaldehyde (1:25 dilution), 0.5 ml. of yeast suspension (0.15 per cent), and 1.5 ml. of distilled water. The experimental mixtures were incubated at 30° for various lengths of time, at the end of which 5 ml. of 20 per cent perchloric acid were added to stop the reaction. Each measurement was corrected with a proper blank in which the addition of cinnamaldehyde was withheld until perchloric acid had been added at the end of the incubation period. Yeast cells were removed by centrifugation, and the ultraviolet absorption spectra of the supernatant fractions were measured. The difference between each incubated supernatant fraction and its respective blank is plotted in Fig. 2 which shows the change in the ultraviolet absorption spectrum during the incubation period. The progressive decline of the cinnamaldehyde absorption maximum at 290 to 295 mμ accompanies a simultaneous increase in the peak at 245 to 250 mμ. There is no increase in the cinnamic acid absorption peak at 271 mμ, which indicates that, if the oxidation of cinnamaldehyde to cinnamic acid occurs during fermentation, none of the latter accumulates. The increase in the peak at 245 to 250 mμ must be due in part to styrene, the absorption maximum of which is at 248 mμ. Cinnamyl alcohol might contribute to part of this peak, because a similar increase in the peak at 245 to 250 mμ was also observed with another strain of yeast which did not convert cinnamaldehyde to styrene.

It was realized that the increase, if any, of the cinnamic acid absorption peak at 271 mμ during incubation could have been masked by the change of absorption of other components. To prove further that no cinnamic acid was accumulated in the fermented medium, 500 ml. of the fermented synthetic medium were acidified and extracted with ether continuously for 48 hours. The ether fraction was then extracted with 5 per cent NaOH solution. The aqueous alkali solution was made acidic with concentrated HCl and reextracted with ether. A brownish oily substance was left after the evaporation of the ether. The ultraviolet absorption spectrum of the

residue was measured in absolute ethanol. No absorption peak at 271 mμ was observed.

A similar experiment in which the fermentation medium was prepared with 0.25 per cent of sodium cinnamate instead of cinnamaldehyde also resulted in the formation of styrene by this strain. After the completion of the fermentation, the fermented medium was adjusted to pH 11.8 with NaOH and steam-distilled. The ultraviolet absorption spectrum of the neutralized steam distillate showed a conspicuous peak of cinnamaldehyde at 291 mμ. It is believed, therefore, that in this case the reduction of cinnamic acid to cinnamaldehyde is involved.

Related saturated compounds, such as hydrocinnamic aldehyde, hydrocinnamic acid, and hydrocinnamic alcohol, exhibit an absorption peak at

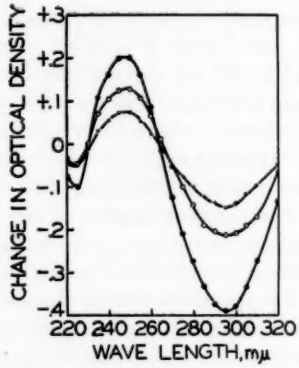
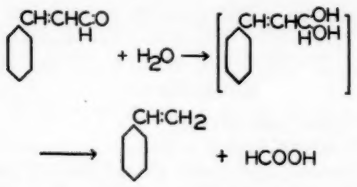


FIG. 2. Change of ultraviolet absorption spectrum during incubation of strain 3212 with cinnamaldehyde for 10 minutes (×), 30 minutes (○), and 60 minutes (●).

260 mμ, but no significant increase of absorption at this wave-length was detected during incubation. It is evident that the hydrogenation of the α,β double bond of cinnamaldehyde was not involved in this conversion of strain 3212.

Another possible mechanism for the conversion of cinnamaldehyde to styrene would be the transient formation of cinnamaldehyde hydrate followed by cleavage to styrene and formic acid. If this mechanism is involved, it might be possible to measure the formic acid produced during fermentation because formic dehydrogenase and hydrogenylase activity had been found absent in this strain. The detection of formic acid in the distillate from the fermented medium containing cinnamaldehyde was attempted with a microcolorimetric method based upon the chromotropic acid-formaldehyde reaction (1). With this method, 2 ml. samples were first acidified with 1 ml. of 2 N HCl, after which 25 mg. of Mg powder were



added. After reduction had been completed, 5 ml. of H_2SO_4-chromotropic acid reagent (13.3 M, containing 2 gm. of chromotropic acid per 500 ml.) were added and heated in a boiling water bath for 30 minutes. Although the method is particularly applicable to yeast fermentation distillate, since the presence of ethyl alcohol has no effect on color development and formic acid recovery, no free formic acid could be demonstrated.

DISCUSSION

The biochemical reduction of cinnamaldehyde to cinnamyl alcohol by fermenting yeast has long been known (2). Fischer and Wiedemann (3) reported that the cinnamyl alcohol thus formed can be further reduced by fermenting yeast to hydrocinnamic alcohol. Similar hydrogenation of other α,β-unsaturated primary alcohols has been reported (4). However, biochemical oxidation of aldehydes by fermenting yeast is less understood. Black (5) isolated a purified preparation of a pyridine nucleotide-dependent aldehyde dehydrogenase from yeast. It was most active in oxidizing acetaldehyde, but it was rather slow with an aromatic aldehyde, such as benzaldehyde, or an α,β-unsaturated aldehyde, such as crotonaldehyde, and totally inactive with salicylaldehyde.

Our inability to detect cinnamic acid formation during the incubation of strain 3212 with cinnamaldehyde would seem to indicate that, if the oxidation of cinnamaldehyde to cinnamic acid occurs, its decarboxylation cannot be a rate-limiting step in styrene formation. While the fact that the cinnamaldehyde absorption peak appeared in the steam distillate of the fermented medium to which only cinnamic acid had been added does not necessarily indicate that cinnamaldehyde is the intermediate between cinnamic acid and styrene, it does show that cinnamaldehyde was produced from cinnamic acid during fermentation. Failure to detect free formic acid in the distillate of fermented medium does not rule out the direct cleavage of cinnamaldehyde hydrate to styrene and formic acid, because the transfer of formyl groups to compounds such as amino acids is quite conceivable in a biological system.

SUMMARY

A biochemical mutant of *Saccharomyces cerevisiae* (strain 3212) can convert cinnamaldehyde to an unsaturated aromatic hydrocarbon, styrene,

during active fermentation. The possible mechanism of this reaction is discussed.

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FURTHER OBSERVATIONS ON THE BIOSYNTHESIS OF L-ASCORBIC ACID FROM D-GLUCOSE IN THE RAT*

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Previous studies showed that the intact carbon chain of glucose can serve as a precursor for the synthesis of ascorbic acid in the rat (1–3). Evidence for such a precursor relationship came from experiments in which glucose tracers labeled in carbon 1 and carbon 6 were administered to rats, and the L-ascorbic acid isolated from urine was labeled predominantly in carbon 6 and carbon 1, respectively. This type of labeling in the ascorbic acid molecule would be expected if the glucose carbon chain is converted without fragmentation to ascorbic acid. These studies were carried out in rats in which the synthesis of ascorbic acid was markedly accelerated by the administration of the hypnotic drug Chloretone. No information was available, however, on the precursors of ascorbic acid in the normal rat. For this reason, in the present study the incorporation of glucose-1-C^{14} into urinary ascorbic acid was compared in normal and Chloretone-treated rats. The results presented in this paper indicate that glucose is a precursor of ascorbic acid in normal rats as well as in Chloretone-treated rats.

EXPERIMENTAL

Radioactive Material—Glucose-1-C^{14} was obtained from the National Bureau of Standards, Washington, D. C., and had a specific activity of 1.0 μc. per mg.

Experimental Animals—Male albino rats of the Wistar strain, weighing 250 to 300 gm., were maintained on a basal diet of evaporated milk for 8 days before each experimental period. The Chloretone-treated animals were given 45 mg. of the drug by stomach tube as a homogenate in 1 ml. of evaporated milk daily for at least 4 days prior to the injection of the labeled glucose.

Isolation Procedures—Urine samples were preserved in 5 ml. of 10 per cent oxalic acid and the amount of ascorbic acid excreted was determined by titration with indophenol dye (4). The ascorbic acid was separated

* This investigation was supported in part by a grant from the Josiah Macy, Jr., Foundation, New York.

from each urine sample after addition of a weighed quantity of carrier ascorbic acid (150 to 300 mg.) by an ion exchange method (4). The ascorbic acid was isolated either as the crystalline compound or as its 2,4-dinitrophenylosazone derivative: the former was recrystallized from a methanol-ether-ligroin solvent mixture and the latter from an acetone-alcohol solvent mixture (4).

Degradation Procedures—L-Ascorbic acid was degraded as follows: carbon 1 was obtained as CO_2 by decarboxylation of L-ascorbic acid with hot mineral acid and carbon 6 was obtained as formaldehyde by periodic acid degradation of its 2,4-dinitrophenylosazone derivative (3).

Radioactive Determination—Preparation of samples and their assay for C^{14} were carried out as described previously (4).

Radioactive Experiments—The conversion of D-glucose-1-C^{14} to ascorbic acid in normal rats was determined in three experiments, two animals being

TABLE I

Radioactive Purity of L-Ascorbic Acid Isolated from Urine of Normal Rats

Collection period	No. of crystallizations	Specific activity	
		Ascorbic acid	Osazone*
hrs.		*c.p.m. per mmole*	*c.p.m. per mmole*
0–24	4	3270	3760
24–72	3	3640	3350

* 2,4-Dinitrophenylosazone derivative of L-ascorbic acid.

employed in each. In Experiment R-1, each rat received 25 mg. of glucose-1-C^{14} by intraperitoneal injection, and urine was collected from 0 to 24 and 24 to 72 hours after the dose. The urine samples obtained from both rats during each collection period were pooled, and the ascorbic acid was isolated both as the crystalline compound and as its 2,4-dinitrophenylosazone derivative. Radioactive purity was established by finding constant specific activity of the osazone and of crystalline ascorbic acid on successive crystallizations. In addition, both the ascorbic acid and its osazone derivative had about the same molar specific activity (Table I). The two other experiments with normal rats (Experiments R-2 and R-3) were carried out in the same manner as Experiment R-1, except that urine samples were collected for a single 24 hour period after administration of glucose-1-C^{14}.

The conversion of D-glucose-1-C^{14} to ascorbic acid in Chloretone-treated rats was determined in three animals. In Experiments R-4 and R-5, each rat received 10 mg. of glucose-1-C^{14} by intraperitoneal injection, and urine samples were collected from 0 to 2.5 and from 2.5 to 24 hours after the

dose.[1] Ascorbic acid was isolated from each urine sample as its 2,4-dinitrophenylosazone derivative which was recrystallized to constant specific activity. In Experiment R-6 the rat was fasted for 24 hours prior to the experiment and received 4.4 mmoles of non-radioactive glucose by stomach tube immediately before the intraperitoneal injection of 10 mg. of glucose-1-C^{14}. The procedure used in the remainder of the experiment was identical to that in Experiments R-4 and R-5, except that urine samples were collected from 0 to 4 and from 4 to 24 hours after the dose.[1]

RESULTS AND DISCUSSION

The amounts of C^{14} incorporated in urinary ascorbic acid, during the 24 hour period after administration of D-glucose-1-C^{14} to normal rats, ranged from 0.003 to 0.016 per cent of the administered C^{14} (Table II). In Experiment R-1, an additional 0.013 per cent of the dose was recovered in the urinary ascorbic acid during the 24 to 72 hour period. The values obtained for the per cent incorporation of D-glucose-1-C^{14} in normal animals are considerably below those reported when the tracer was administered to Chloretone-treated animals which averaged about 0.5 per cent of the dose (2). This is expected, since normal animals excrete only 1 to 2.6 mg. of ascorbic acid daily in the urine compared to 30 to 40 mg. for Chloretone-treated rats. The marked increase in urinary excretion of ascorbic acid in Chloretone-treated rats has been shown previously to result from an increased rate of ascorbic acid biosynthesis (4).

TABLE II

Incorporation of C^{14} in Urinary Ascorbic Acid after Administration of D-Glucose-1-C^{14} to Normal Rats

Experiment No.	Time	Ascorbic acid excretion*	Per cent of dose in ascorbic acid	Per cent of total C^{14} in ascorbic acid in carbon 6
	hrs.	*mg. per day*		
R-1	0–24	2.6	0.016	67
	24–72	2.4	0.013	61
R-2	0–24	2.5	0.010	60
R-3	0–24	1.0	0.003	51

* Average ascorbic acid excretion per rat in each experiment.

The fraction of the total C^{14} in ascorbic acid found in carbon 6 varied

[1] In order to insure adequate urine sampling during the initial collection period, the animals were given 5 ml. of water by stomach tube 90 and 30 minutes before injecting the labeled compound. At the end of the collection period the animals were made to urinate by allowing them to inhale ether vapors.

from 51 to 67 per cent in the normal rat (Table II). In Experiment R-1 the ascorbic acid isolated during the 0 to 24 and the 24 to 72 hour periods was found to have about 10 per cent of its total C^{14} in carbon 1. The finding that the major fraction of the C^{14} in ascorbic acid was present in carbon 6 is similar to the results reported previously when glucose-1-C^{14} was administered to Chloretone-treated rats (2, 3). In this previous study, the ascorbic acid isolated from urine samples collected over a 24 hour period after the dose contained about 56 per cent of its total C^{14} in carbon 6 and about 10 per cent in carbon 1. Since there is a similar distribution of C^{14} in ascorbic acid isolated from the urine of normal rats and of Chloretone-treated rats, it appears that the pathway of biosynthesis in which the intact carbon chain of glucose is employed is the same for both groups of animals.

TABLE III

Incorporation of C^{14} in Urinary Ascorbic Acid after Administration of D-Glucose-1-C^{14} to Chloretone-Treated Rats

Experiment No.	Time	Ascorbic acid excretion	Per cent of dose in ascorbic acid	Per cent of total C^{14} in ascorbic acid in carbon 6
	hrs.	*mg.*		
R-4	0– 2.5	4.7	0.07	76
	2.5–24	40.2	0.43	64
R-5	0– 2.5	3.1	0.03	75
	2.5–24	41.4	0.37	61
R-6*	0– 4	6.5	0.04	78
	4–24	22.4	0.10	62

* The rat was fasted for 24 hours prior to the experiment and received 4.4 mmoles of non-radioactive glucose by stomach tube immediately before injection of labeled glucose.

The administration of glucose-1-C^{14} would be expected to give rise to ascorbic-6-C^{14} acid if the glucose carbon chain is converted intact to ascorbic acid. The results obtained in normal and Chloretone-treated rats indicate that, although carbon 6 of ascorbic acid synthesized over 24 hours contains the major fraction of the total C^{14} in the molecule, appreciable radioactivity is also present in the other 5 carbon atoms. In order to obtain additional information on the conversion of glucose to ascorbic acid, the incorporation of C^{14} into urinary ascorbic acid was measured at different time intervals after administration of glucose-1-C^{14} to Chloretone-treated rats (Table III). In Experiments R-4 and R-5, the ascorbic acid collected during the 0 to 2.5 hour period contained an average of 76 per cent of the total C^{14} in carbon 6 compared to an average of 63 per cent for the ascorbic acid collected

during the 2.5 to 24 hour period. In Experiment R-6 the ascorbic acid collected during the 0 to 4 hour period contained 78 per cent in carbon 6 compared to 62 per cent in the ascorbic acid collected during the 4 to 24 hour period. These results, that the ascorbic acid synthesized within a few hours after the dose had a greater fraction of the C^{14} in carbon 6 than that synthesized over a 24 hour period, can be explained by a partial redistribution of the C^{14} in the glucose pool during the 24 hour period within which glucose is converted to ascorbic acid. Accordingly, it would be expected that the fraction of the C^{14} in carbon 1 of glucose would be maximal during the early period immediately after injection of glucose-1-C^{14}, resulting in the formation of ascorbic acid labeled mainly in carbon 6. Other examples of such "randomization" of D-glucose-1-C^{14} in the body pool have recently been reported for the synthesis of glycogen in fasted rats (5) and for the synthesis of menthyl glucuronide in rabbits (6). The present experiments, in which the incorporation of glucose-1-C^{14} into urinary ascorbic acid was measured at different times, furnish further evidence for the direct utilization of the intact carbon chain of glucose in the synthesis of ascorbic acid.

SUMMARY

The incorporation of C^{14} into urinary ascorbic acid was compared in normal and Chloretone-treated rats after the administration of D-glucose-1-C^{14}. The major fraction of the total C^{14} in the isolated ascorbic acid was present in carbon 6, furnishing evidence that the intact carbon chain of glucose is utilized for the synthesis of ascorbic acid in the normal rat as well as in the Chloretone-treated rat.

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CARBOXYMETHYLAMINO ACIDS AND PEPTIDES*

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The valuable method of Sanger (1) for the labeling of terminal amino groups in peptides by means of dinitrofluorobenzene suffers from the disadvantage that acid hydrolysis of the products is frequently attended by extensive decomposition of the resulting dinitrophenylamino acids (2). The study here reported was undertaken in order to explore the stability, under conditions of acid hydrolysis, of *N*-carboxymethyl (Cm-)[1] derivatives of amino acids, and the feasibility of their separation from natural amino acids. In both respects the results offer promise.

In spite of our failure to include all natural amino acids in the survey and to obtain every product in crystalline form, we now present the results in the hope that they may find application in other laboratories.

Cm-sarcosine (3) and several α,α'-iminodicarboxylic acids such as octopin (4) and mono-Cm-alanine (5) and its homologues (6–9) have been described in the literature, but di-Cm-amino acids other than di-Cm-glycine (10) have received comparatively little attention. Michaelis and Schubert (11) prepared acid potassium salts of di-Cm-alanine, tetra-Cm-cystine, and *N*-di-Cm-cysteine. They also described the preparation of *S*-Cm-cysteine by brief treatment of an alkaline solution of cysteine with potassium chloroacetate; analogous reactions of cysteine with neutral iodoacetate had previously been recorded by Dickens (12) and Rapkine (13).

Cm-amino Acids

The carboxymethyl group is conveniently introduced by treating amino acids with magnesium bromoacetate in an aqueous suspension of magnesium oxide. In most instances, when an excess of the reagent is employed, two Cm- groups attach themselves to the α-amino nitrogen atom, the chief exception, besides proline, being aspartic acid, from which the mono-Cm-derivative was preponderantly formed under the conditions employed. Glutamic acid and phenylalanine yielded some mono-Cm- as well as di-Cm- derivatives. Tyrosine gave two derivatives, in both of which the

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[1] Hereafter referred to as Cm-.

XUM

phenolic hydroxyl group had also been carboxymethylated. The substitution of the hydroxylic hydrogen atom of tyrosine was accompanied (Fig. 1) by abolition of the shift in the ultraviolet absorption spectrum caused by alkali, characteristic of phenols (14). However, alkali induces a general increase in absorption in the region of the maximum and at shorter wavelengths. A similar effect was observed (Fig. 2) with phenylalanine.

As was to be expected, the guanidino group of arginine does not react with bromoacetate; lysine, on the other hand, yields a tetra-Cm- derivative.

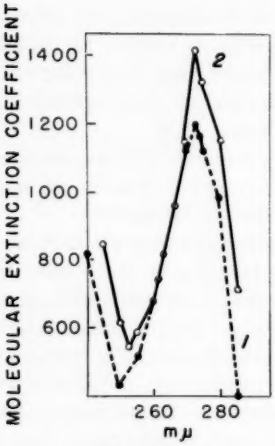


FIG. 1

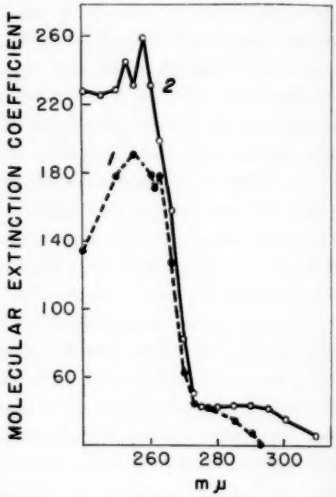


FIG. 2

FIG. 1. Absorption spectra of tri-Cm-tyrosine. Curve 1, solvent, 0.1 N HCl; Curve 2, solvent, 0.1 N NaOH.

FIG. 2. Absorption spectra of phenylalanine. Curve 1, solvent, 0.1 N HCl; Curve 2, solvent, 0.1 N NaOH.

Attempts were made to prepare ε-di-Cm-lysine by the action of bromoacetate upon the copper complex of lysine in a procedure analogous to that devised by Kurtz (15) for the synthesis of ε-carbobenzyloxylysine. These were unsuccessful; substitution occurred at the α-amino group as well as at the terminal nitrogen atom. The desired compound, however, was secured, in the form of its mercuric derivative, from alanyllysylalanine.

When treated with bromoacetate in the presence of MgO, cysteine and histidine, like tyrosine, react in their specific functional groups. The reaction with the sulfhydryl group of cysteine is very rapid, as was found by Huggins and Jensen (16) in the case of iodoacetate; with histidine and

tyrosine the ability to yield color with diazobenzenesulfonic acid disappeared at measurable rates (Table I). Iodoacetate can be employed in place of bromoacetate for the carboxymethylation of amino acids, but its action is less rapid; chloroacetate is even less effective.

The products obtained from all of the amino acids investigated have been found, irrespective of the number of carboxymethyl groups introduced, to be precipitable by means of mercuric nitrate under conditions which lead to the precipitation of none of the natural amino acids except cysteine, cystine, methionine, histidine, tryptophan, and aspartic acid; these, however, unlike the α-Cm-amino acids, are precipitable by mercuric acetate

TABLE I

Reaction of Amino, Phenolic, and Imidazole Groups with Haloacetates

Time	Chloroacetate			Bromoacetate			Iodoacetate		
	Tyrosine		Histidine	Tyrosine		Histidine	Tyrosine		Histidine
	NH_2	Phenol	Imidazole	NH_2	Phenol	Imidazole	NH_2	Phenol	Imidazole
hrs.									
3	6	0	1	72	8	18	59	2	14
7	9	0	2	100			95		
16					44	60			
24	35	0	6		55	74		11	50
48	56	0	11		76	95		23	74
72	76	0	14		87	100		26	86

The reaction mixtures, 0.025 M in amino acid and 0.215 M in haloacetate, were gently agitated at 35–37° with an excess of MgO. The values shown indicate the per cent disappearance of amino nitrogen and groups which yield color with diazobenzenesulfonic acid.

in the presence of 0.1 M sodium chloride. The mercuric derivatives of the α-Cm-amino acids melt, with decomposition, at definite temperatures (Table II). They readily dissolve in dilute solutions of sodium chloride or hydrochloric acid containing at least one chlorine ion for every atom of mercury; the resulting soluble complexes presumably contain the grouping —COOHgCl, in analogy with the formulation suggested by Toennies and Kolb (17) in their study of the action of mercuric chloride on methionine. On the addition of excess of mercuric nitrate to such solutions, the derivatives are reprecipitated. In general, they appear to contain 1 atom of mercury for every two carboxyl groups.

The calcium salts of Cm-amino acids are, like calcium aspartate and glutamate, readily soluble in water but insoluble in alcohol. The same is true of the magnesium salts.

Titration curves, typified by Figs. 3 and 4, indicate that the introduction of carboxymethyl groups has, as was shown by Michaelis and Schubert in the case of di-Cm-glycine (11), little effect on the basic function of the

TABLE II

Melting Decomposition Points of Hg Derivatives of Carboxymethylamino Acids Precipitated by Mercuric Nitrate

	°C.		°C.
Di-Cm-glycine	186–187	Di-Cm-glutamic acid	174–175
Di-Cm-alanine	177–178	Di-Cm-serine	168–170
Di-Cm-valine	163–164	*O,N*-Di-Cm-tyrosine	192–194
Di-Cm-leucine	169–171	Tri-Cm-tyrosine	186–189
Di-Cm-isoleucine	157–158	Tetra-Cm-lysine	160–162
Mono-Cm-phenylalanine	180–182	Di-Cm-arginine	185–188
		Di-Cm-histidine	203
Di-Cm-phenylalanine	174–175	Tri-Cm-histidine	213–218
Cm-proline	178–180	*N,S*-Di-Cm-cysteine	165–173
Mono-Cm-aspartic acid	181–184	Di-Cm-methionine	128
Mono-Cm-glutamic acid	174–176		

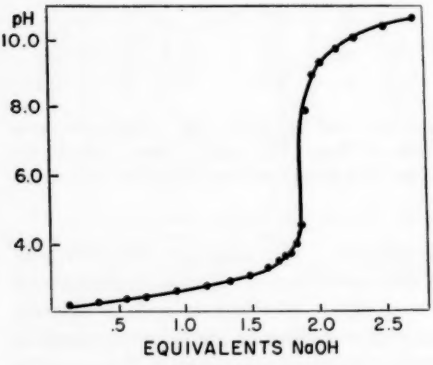


FIG. 3

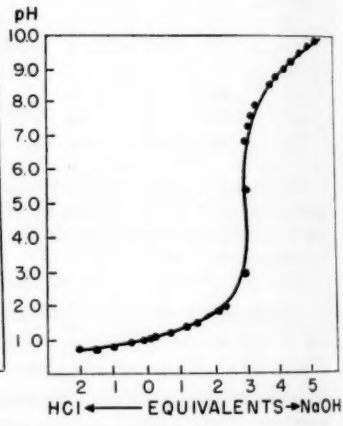


FIG. 4

FIG. 3. Titration curve of di-Cm-leucine
FIG. 4. Titration curve of tetra-Cm-lysine

nitrogen. Over the pH range 4 to 8, the compounds exhibit little buffering power and must therefore exist in essentially dipolar ionic form.

The optical rotations of the Cm-amino acids in water, acid, and alkali, three of which are recorded in Table III, suggest that dextrorotational minima exist, as is the case (18) with natural amino acids, in their non-buffering regions.

In many instances, individual Cm-amino acids can be separated by paper chromatography. The R_F values, obtained by the conventional method of upward migration, with a solvent consisting of a mixture of butanol, ethanol, and aqueous ammonia, are shown in Table IV. Identical results were obtained with saline solutions of the mercury derivatives, the metallic constituent of which is detached, presumably by the ammonia, and remains immobile. The di-α-Cm-amino acids yielded dark purple spots with the reduced ninhydrin reagent of Long, Quayle, and Stedman (19), whereas the colors given by mono-α-Cm-amino acids had a pinkish purple hue.

TABLE III

Specific Rotations $[\alpha]_D^{27}$ *in Water, Acid, and Alkali*

	Water	N-HCl	N-NaOH
	degrees	*degrees*	*degrees*
Di-Cm-leucine	+2.6	+19	+15
Di-Cm-phenylalanine	+3.5	+24	+28
Mono-Cm-aspartic acid	+4.1	+11	+11

TABLE IV

R_F Values (±0.03) of Carboxymethylamino Acids

Di-Cm-glycine	0.37	Di-Cm-serine	0.33
Di-Cm-alanine	0.48	*O*-Mono-Cm-tyrosine	0.44
Di-Cm-valine	0.48	Tri-Cm-tyrosine	0.36
Di-Cm-leucine	0.73	Di-Cm-lysine	0.32
Di-Cm-isoleucine	0.54	Tetra-Cm-lysine	0.27
Mono-Cm-phenylalanine	0.62	Di-Cm-arginine	0.28
Di-Cm-phenylalanine	0.62	Di-Cm-histidine	0.27
Cm-proline	0.45	Tri-Cm-histidine	0.32
Mono-Cm-aspartic acid	0.35	Di-Cm-methionine	0.37
Mono-Cm-glutamic acid	0.38		

Some Cm-amino acids can be extracted from solution in 0.1 N hydrochloric acid by ether or ethyl acetate. When a group of markedly hydrophobic character is present in the molecule, as in the derivatives of leucine and phenylalanine (Table V), the distribution coefficient may exceed unity.

In this admittedly incomplete study of the carboxymethylation of amino acids, we were unable to secure demonstrably homogeneous products from serine, threonine, cystine, and tryptophan. Further work on these is necessary.

Cm- Peptides

A few typical synthetic peptides have been carboxymethylated under similar conditions. The products, which likewise formed insoluble com-

plexes on treatment with mercuric nitrate, after acid hydrolysis gave the Cm- derivatives of the *N*-terminal amino acids in yields, in most cases, of over 90 per cent.

The isolation of the Cm- derivatives of the terminal amino acids is complicated by the fact that a few natural amino acids (see above) yield insoluble mercuric salts with mercuric nitrate. The same is true of compounds such as ϵ-di-Cm-lysine or *O*-Cm-tyrosine, in which the α-amino groups are unsubstituted. However, all of these, with the exception of histidine carboxymethylated exclusively in the imidazole ring, are precipitated by the addition of mercuric acetate to their solutions in 0.1 to 0.5 M NaCl. Under these conditions, none of the α-Cm-amino acids hitherto examined is precipitated by mercuric acetate. On the other hand, aspartic acid and glu-

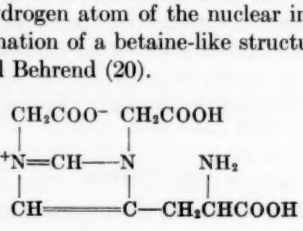
TABLE V

Distribution Ratios, (N per Ml. of Solvent)/(N per Ml. of 0.1 N HCl)

Compound	Ether	Ethyl acetate	Compound	Ether	Ethyl acetate
Di-Cm-glycine	0.0	0.0	Di-Cm-glutamic acid	0.029	0.074
Di-Cm-alanine	0.01	0.04	Di-Cm-serine		0.02
Di-Cm-valine	0.29	1.25	*O*-Mono-Cm-tyrosine	0	0.007
Di-Cm-leucine	0.61	1.88	*O,N*-Di-Cm-tyrosine	0.0	0.01
Di-Cm-isoleucine	0.67	1.75	Tri-Cm-tyrosine	0.05	1.00
Mono-Cm-phenylalanine	0.0	0.01	Tetra-Cm-lysine	0.00	0.00
Di-Cm-phenylalanine	1.02	3.85	Di-Cm-arginine	0.00	0.00
Cm-proline	0.0	0.0	Tri-Cm-histidine	0.00	0.01
Mono-Cm-aspartic acid	0.0	0.01	Di-Cm-methionine	0	0
Mono-Cm-glutamic acid	0	0			

tamic acid are precipitated by mercuric acetate when the chloride concentration is less than 0.2 M, and the mercuric salt of imidazole-di-Cm-histidine is quantitatively precipitated on the addition of an equal volume of ethanol.

In the di-Cm- derivative of histidine mentioned above, produced from glycyl- and alanylhistidine with subsequent hydrolysis, one of the Cm-groups replaces the hydrogen atom of the nuclear imino group; the other contributes to the formation of a betaine-like structure, analogous to that described by Rung and Behrend (20).



Histidine itself appears, under the experimental conditions, to accept the second carboxymethyl group on the imidazole nucleus with greater difficulty, if at all.

EXPERIMENTAL

Carboxymethylation of Amino Acids

In general, the reaction mixtures, consisting of an amino acid (0.025 M), magnesium bromoacetate (0.2 M), and an excess of MgO were gently agitated at 35° for 3 days. Unless otherwise indicated, the solutions were filtered and acidified to Congo red; saturated mercuric nitrate solution was then added until no further precipitation occurred. Purification of the mercuric derivatives was effected by solution in 0.05 M NaCl and reprecipitation with mercuric nitrate. The precipitates were washed with water until free of nitrate, and decomposed with H_2S. The resulting solutions were concentrated under reduced pressure to approximately 1 M, adjusted to pH 1 to 1.5 with NaOH, and the products isolated by the procedures described below.

Analytical estimations of mercury and nitrogen in the mercuric derivatives were conveniently carried out as follows. A weighed sample (30 to 50 mg.) was heated with 0.2 ml. of concentrated sulfuric acid in a tared centrifuge tube. When the mixture was nearly colorless (generally after 20 minutes), it was allowed to cool, diluted with 10 ml. of water, and saturated with hydrogen sulfide. The precipitate of HgS was well washed and dried to constant weight at 105°. The supernatant solution and washings were united, concentrated, and subjected to Kjeldahl analysis.

Di-Cm-alanine—The solution (5 ml.) from a 3 mmole preparation was adjusted to pH 1.3 and shaken with three 100 ml. portions of ethyl acetate. The extract, which contained 2.2 m.eq. of N, was taken to dryness under reduced pressure, 20 ml. of absolute alcohol were added, and the suspension was again evaporated *in vacuo*. The residue was dissolved in 20 ml. of hot 95 per cent ethanol, the solution was allowed to cool, and the 258 mg. of product (1.2 m.eq. of N) which separated were recrystallized from 95 per cent ethanol. It melted at 208° with decomposition.

$C_7H_{11}O_6N$. Calculated, C 41.0, H 5.4, N 6.8; found, C 41.0, H 5.4, N 7.2

Di-Cm-valine—The solution (3 ml.) from a 3 mmole preparation at pH 1.6 was shaken with five 12 ml. portions of ethyl acetate. The extract (2.0 m.eq. of N) was concentrated to a syrup and dissolved in 1 ml. of hot ethyl acetate. The solution, which crystallized on cooling, was treated with 2 ml. of ether and allowed to stand for a few days. The dried product weighed 217 mg. (0.93 m.eq. of N). After purification by solution in

1 ml. of water, repetition of the extraction, and recrystallization from ethyl acetate, it melted at 176°.

$C_9H_{15}O_6N$. Calculated, C 46.4, H 6.4, N 6.0; found, C 46.6, H 6.6, N 6.1

Di-Cm-leucine—The solution (7 ml.) from a 9 mmole preparation was allowed to stand for several days at 0–5°. The solid which separated was recrystallized three times from water. The product weighed 1.285 gm. (58 per cent of the theoretical amount), melted at 178°, and had $[\alpha]_D$ +18° (1.3 per cent in 0.5 N HCl).

$C_{10}H_{17}O_6N$. Calculated, C 48.6, H 6.9, N 5.7; found, C 48.4, H 6.7, N 5.6

The titration curve is shown in Fig. 3.

The mercury derivative gave satisfactory analytical values for N and Hg, but contained a small amount of Cl.

$C_{20}H_{28}O_{12}N_4Hg_3$. Calculated, N 2.6, Hg 55.4, Cl 0.0; found, N 2.5, Hg 55.2, Cl 1.9

The precipitability of the calcium salt is indicated by the following experiment. To a solution of 50 mg. (0.20 mmole) of di-Cm-leucine in 5 ml. of water, calcium hydroxide was added in excess. The alkaline suspension was centrifuged, the clear solution treated with 25 ml. of ethanol, and the mixture allowed to stand overnight in the refrigerator. After centrifugation, the supernatant solution was found to contain 0.018 m.eq. of N.

Di-Cm-isoleucine—The solution from a 3 mmole preparation was shaken with four 20 ml. portions of ethyl acetate; the extract (2.4 m.eq. of N) was taken to dryness *in vacuo*; the residue was dissolved in 3 ml. of warm ethyl acetate and the product precipitated by the addition of 3 ml. of light petroleum. After a second such reprecipitation, the yield was 251 mg. of an amorphous product melting at 121°. After three more such precipitations the melting point was 123–124°. This product was not obtained in crystalline condition.

$C_{10}H_{17}O_6N$. Calculated, C 48.6, H 6.9, N 5.7; found, C 48.3, H 7.0, N 5.7

Mono-Cm-phenylalanine—The carboxymethylation mixture from a 3 mmole preparation was filtered and allowed to stand for a month in the refrigerator. The magnesium salt of the monosubstitution product, which separated in crystalline form, was washed with ice-cold water and dried at 120°. The yield was 150 mg.

$C_{22}H_{24}O_8N_2Mg \cdot 4H_2O$. Calculated. C 48.8, H 5.9, N 5.2, Mg 4.5
Found. " 48.6, " 5.7, " 5.2, " 4.1

A 25 mg. portion of this salt was dissolved in 2.5 ml. of 0.1 N HCl. Crystallization of the free acid began almost immediately. After 24 hours at

0–5°, the product was collected, washed, and dried over P_2O_5 *in vacuo* at room temperature. Yield, 17 mg.; m.p. 228–233°, with decomposition.

$C_{11}H_{13}O_4N$. Calculated, N 6.3; found, N 6.2

Di-Cm-phenylalanine—The original filtrate from the above magnesium salt was acidified to Congo red with HCl and treated with 35 ml. of saturated mercuric nitrate solution. The precipitate was washed and dried; weight 1.935 gm. (2.3 m.eq. of N). The concentrated filtrate (8 ml.) obtained after removal of mercury as sulfide was adjusted to pH 1 and allowed to stand for a few days in the refrigerator. The crystalline di-Cm-phenylalanine weighed 405 mg. After three recrystallizations from water it melted at 141° and had $[\alpha]_D$ +21° (1.4 per cent in 0.5 N HCl).

$C_{13}H_{15}O_6N$. Calculated, C 55.5, H 5.3, N 5.0; found, C 55.3, H 5.6, N 4.8

Cm-proline—The solution (2.5 ml.) from a 3 mmole preparation was treated with 15 ml. of acetone and cooled at 0–5° overnight. The syrupy precipitate was washed with 3 ml. of acetone, dissolved in 2 ml. of water, and treated with 1 ml. of methanol and 13 ml. of isopropanol. The crystalline product, weighing 231 mg. (1.3 m.eq. of N), was twice recrystallized from water with the addition of isopropanol. It melted at 220° (decomposition).

$C_7H_{11}O_4N$. Calculated, C 48.5, H 6.4, N 8.1; found, C 48.8, H 6.3, N 7.8

Mono-Cm-aspartic Acid—The reaction mixture from 1.000 gm. of aspartic acid (7.5 mmoles), 5.0 gm. of bromoacetic acid (36 mmoles), and excess of magnesium oxide in 75 ml. of water was filtered, concentrated at about 30° to 60 ml., and treated with 300 ml. of absolute ethanol. The precipitate was washed with 95 per cent ethanol. The filtrate and washings, which contained magnesium bromide, unchanged bromoacetate, and only 0.18 m.eq. of N, were discarded. The solid was dissolved in water, acidified to Congo red, and treated with an excess of mercuric nitrate solution. A portion of the precipitate was dissolved in NaCl, reprecipitated, and dried at 100° for analysis.

$C_{12}H_{12}O_{12}N_2Hg_3$. Calculated, N 2.87, Hg 61.5; found, N 2.73, Hg 60.5

The remainder was suspended in water and decomposed with hydrogen sulfide. The filtrate, containing 6.4 m.eq. of N, was evaporated to dryness under reduced pressure, redissolved in 2 ml. of water, treated with 20 ml. of acetone, and allowed to stand overnight. The gummy precipitate, which had become microcrystalline and friable, was washed first with acetone containing 10 per cent of water, then with pure acetone. The filtrate and washings were evaporated to dryness and the residue was again treated

with cold aqueous acetone, when a small second crop was secured. The combined product weighed 695 mg. (3.6 mmoles). On recrystallization by addition of isopropanol to a concentrated aqueous solution, it formed rectangular needles which melted at 198–199°.

$C_6H_9O_6N$. Calculated, C 37.7, H 4.7, N 7.3; found, C 37.8, H 4.7, N 7.2

A titration curve, determined in 0.1 M NaCl, showed the presence of three acid groups with apparent pK values of approximately 2, 2.4, and 3.9, respectively, and one basic group, pK 9.6.

In a similar preparation, the concentrated filtrate from the HgS was adjusted to pH 1.3 with sodium hydroxide before the addition of acetone. The product in this case was a crystalline, hydrated monosodium salt, m.p. 162°, $[\alpha]_D$ +11.4° (1.2 per cent in 0.5 N HCl).

$C_6H_8O_6NNa \cdot H_2O$. Calculated. C 31.3, H 4.3, N 6.1, Na 10.0
Found. " 31.8, " 4.3, " 6.1, " 9.7

Di-Cm-aspartic acid appeared to be formed, as a minor product, when aspartic acid was treated with a larger excess of bromoacetate for a longer time and at a higher temperature: a mixture of 1.000 gm. of aspartic acid (7.5 mmoles), 10 gm. of bromoacetic acid (72 mmoles), and 5 gm. of magnesium oxide in 100 ml. of water was stirred for a week at 37–42°, filtered, concentrated at 90° to a total weight of 30 gm., treated with 150 ml. of absolute ethanol, and allowed to stand at 3–5° for 3 days. The precipitate was collected and washed with ethanol. The filtrate and washings, which contained 0.13 m.eq. of N, were discarded; the solid product was dissolved in water, freed of alcohol by boiling, and acidified to pH 1 with 10 ml. of 5 N H_2SO_4. This solution was subjected to continuous extraction with ethyl acetate for four successive 10 hour periods, during which 28, 4.3, 1.8, and 1.8 mg. of N, respectively, were extracted. The first extract, which weighed 2.7 gm. and contained much glycolic acid, was dissolved in water and treated with mercuric nitrate; 1.07 gm. of a mercuric derivative, m.p. 181–188°, were precipitated.

$C_8H_7O_8NHg_2$. Calculated, N 2.17, Hg 62.0; found, N 2.00, Hg 62.4

The acid solution remaining after the extraction yielded a mercuric salt which appeared to consist mainly of the mono-Cm- derivative (found, N 2.37, Hg 60.2).

Mono-Cm-glutamic Acid—The solution (5 ml.) from a 3 mmole preparation was adjusted to pH 1.2 and the product extracted with seven 20 ml. portions of ethyl acetate. To the aqueous phase, which contained 0.79 m.eq. of N, isopropanol (6 ml.) was added until a slight cloudiness appeared. The mixture, after a few days in the refrigerator, deposited crystals (30

mg.) which were redissolved in water (2 ml.) and treated with isopropanol (7 ml.); crystallization occurred almost immediately; m.p. 155–156°.

$C_7H_{11}O_6N$. Calculated, C 41.0, H 5.4, N 6.8; found, C 41.2, H 5.4, N 6.9

Di-Cm-glutamic Acid—The above ethyl acetate extract, which contained 1.54 m.eq. of N, was evaporated to dryness under reduced pressure, redissolved in 5 ml. of hot ethyl acetate, and precipitated with light petroleum. This process was repeated. The resulting 270 mg. of amorphous product were crystallized from 3 ml. of hot ethyl acetate, when 15 mg. of crystalline, very hygroscopic di-Cm-glutamic acid were secured; m.p. 85°.

$C_9H_{13}O_8N$. Calculated, C 41.1, H 4.9, N 5.3; found, C 40.8, H 5.1, N 5.5

A titration curve indicated the presence of five dissociable groups, of approximate pK values 2, 2.4, 3, 4.5, and 8.5.

Di-Cm-serine—The concentrated filtrate (3 ml.) from a 3 mmole preparation was brought to pH 1.3 with 0.15 ml. of 11 N NaOH, treated with 6 ml. of acetone, and allowed to stand overnight in the refrigerator. The precipitated syrup was washed with 3 ml. of acetone and dissolved in 1 ml. of water. Addition of a mixture of 1.5 ml. of methanol and 3 ml. of isopropanol caused the precipitation of a syrup which was redissolved in 1 ml. of water and treated with 9 ml. of methanol. The precipitate, which soon solidified when the mixture was cooled to 5°, was collected after the addition of 3 ml. of ethanol at −10°. After being washed with ethanol and ether, the amorphous product was dried over P_2O_5 at room temperature; m.p. 150–155°. Attempts to crystallize this product failed.

$C_7H_{11}O_7N$. Calculated, N 6.3; found, N 5.7

O-Mono-Cm-tyrosine—A solution of 335 mg. (1.5 mmoles) of *N*-acetyltyrosine was carboxymethylated and the product precipitated with mercuric nitrate in the usual way. After decomposition with hydrogen sulfide, the filtrate was concentrated to 3 ml. and boiled under a reflux for 20 hours with an equal volume of concentrated HCl. The excess of HCl was removed by repeated evaporation under reduced pressure. The residue was dissolved in water (5 ml.), brought to pH 1.7, and chilled overnight in the refrigerator. The crystalline product was washed with ice-cold water, then with acetone, and dried at 110°; yield, 75 mg. After recrystallization from boiling water (9 ml.), it melted, with decomposition, at 260–261°.

$C_{11}H_{12}O_5N$. Calculated, C 55.3, H 5.4, N 5.8; found, C 55.4, H 5.6, N 5.7

The mercury derivative, precipitated on the addition of mercuric acetate, melted at 205–208°.

O,N-Di-Cm-tyrosine—The solution from a 3 mmole preparation was

shaken with six 7 ml. portions of ethyl acetate. During the extraction, a crystalline precipitate formed in the aqueous layer. This was washed with water and dried over P_2O_5; it weighed 90 mg. and contained 0.29 m.eq. of N. After recrystallization from hot water (1.5 ml.), it melted with decomposition at 241–243°.

$C_{13}H_{15}O_7N$. Calculated, C 52.5, H 5.1, N 4.7; found, C 52.5, H 5.3, N 4.8

As this compound gave no color with Millon's reagent, one of the carboxymethyl groups must have been linked to the phenolic oxygen atom.

Tri-Cm-tyrosine—The foregoing ethyl acetate extract was evaporated to dryness and the residue recrystallized from ethyl acetate; yield, 560 mg. After recrystallization from hot water (5 ml.) it melted with decomposition at 183–184°.

$C_{15}H_{17}O_9N$. Calculated, C 50.7, H 4.8, N 3.9; found, C 50.7, H 5.1, N 3.9

Tetra-Cm-lysine—The solution (4 ml.) from a 3 mmole preparation was brought to pH 1.3, treated with 17 ml. of acetone, and chilled at 0–5° for a day. The syrupy precipitate was dissolved in 2 ml. of water, reprecipitated with 5 ml. of isopropanol, and redissolved in 2 ml. of water. The hot solution was treated with 14 ml. of hot methanol and chilled; the solid product, weighing 741 mg. (60 per cent of the theoretical amount), was twice recrystallized from 7 ml. portions of hot water. It melted with decomposition at 220°.

$C_{14}H_{22}O_{10}N_2$. Calculated, C 44.4, H 5.8, N 7.4; found, C 44.4, H 6.0, N 7.4

The titration curve (Fig. 4) indicates the presence of three titratable and two compensated acid groups.

Formation of the copper complex of lysine (15) failed to prevent the reaction of bromoacetate with the α-amino group. A solution of 344 mg. of lysine dihydrochloride in 30 ml. of water was adjusted to pH 8 with sodium hydroxide and heated on the steam bath for 2 hours with excess of basic copper carbonate. The mixture was then filtered. Aliquot portions were treated with magnesium oxide and bromoacetate by the standard procedure, and with sodium bromoacetate in the presence of sodium carbonate at pH 9.5. Addition of 5 volumes of 95 per cent ethanol to the resulting solutions caused the precipitation of blue, amorphous solids or syrups which, after the removal of copper by hydrogen sulfide, gave no color with ninhydrin and no amino nitrogen in the Van Slyke test.

Di-Cm-arginine—The reaction mixture from 1.6 mmoles of arginine and 18 mmoles of bromoacetate in 50 ml. of water was filtered and allowed to stand for 3 weeks in the refrigerator. The crystals which separated weighed

323 mg. and contained 3.6 m.eq. of N. This salt was twice recrystallized from hot water and dried at 130°.

$C_{10}H_{16}O_6N_4Mg$. Calculated, N 17.9, Mg 7.8; found, N 17.8, Mg 7.7

A 250 mg. portion of the Mg salt was dissolved in 20 ml. of 0.05 N HCl and treated with an excess of mercuric nitrate. The washed precipitate was decomposed with H_2S and the filtrate concentrated to 1.5 ml. under reduced pressure. On the addition of 3.5 ml. of isopropanol, crystallization soon began. After 20 hours in the refrigerator, the product was washed with 60 per cent aqueous isopropanol; dried at 110° after recrystallization from water-isopropanol, it weighed 120 mg. and melted at 160–161°.

$C_{10}H_{18}O_6N_4$. Calculated, N 19.3; found, N 19.9

Tri-Cm-histidine—The solution from a 3 mmole preparation was evaporated under reduced pressure and the syrupy residue was dissolved in 10 ml. of acetone. Treatment of this solution with 10 ml. of ethyl ether caused the separation of a liquid which was no longer soluble in acetone. It was dissolved in 2 ml. of water, treated with 15 ml. of isopropanol, and chilled to −10°, when a gummy solid separated. The supernatant solution contained 4.5 m.eq. of N, which corresponded to 50 per cent of the histidine employed. The gum was converted to an amorphous solid by dissolving it in 3 ml. of water and adding 15 ml. of methanol. After being dried over P_2O_5, this product, which gave no color with the Pauly reagent, weighed 124 mg.

$C_{12}H_{15}O_8N_3$. Calculated, N 12.8; found, N 12.2

It was not quite homogeneous, for on subjection to paper chromatography it yielded two spots. The major, more rapidly moving component gave the dark purple color characteristic of the di-α-Cm-amino acids. The minor, slower moving component gave a pinkish color with reduced ninhydrin and therefore appeared to be the α-mono-Cm derivative.

N,S-Di-Cm-cysteine—Carboxymethylation of 3 mmoles of cysteine with 36 mmoles of bromoacetate was carried out for 3 days at 37°. The filtrate from the decomposition of the mercuric derivative was concentrated to 3 ml. and adjusted to pH 1.4, when a crystalline product separated. After being washed with cold water and dried, it weighed 150 mg.; m.p. 237° (decomposition).

$C_7H_{11}O_6NS$. Calculated, N 6.0; found, N 6.4

Extraction of the mother liquor with ethyl acetate yielded a syrup which could not be induced to crystallize.

Di-Cm-methionine—The solution (5 ml.) from a 1.5 mmole preparation

was brought to pH 1.7, treated with 25 ml. of methanol and 25 ml. of isopropanol, and chilled overnight in the refrigerator. The precipitate, which contained 0.61 m.eq. of N, was recrystallized three times from water-isopropanol; yield, 75 mg.; m.p. 168°.

$C_9H_{15}O_6NS$. Calculated, C 40.8, H 5.7, N 5.3; found, C 40.8, H 6.1, N 5.3

Cm-amino Acids from Peptides

The carboxylation of peptides was carried out exactly as with the amino acids. The products likewise yielded insoluble salts on treatment with mercuric nitrate but, as the precipitates could not be completely freed from nitrate ion by washing, they were decomposed with H_2S and the calcium salts, precipitated by addition of alcohol, were subjected to acid hydrolysis. After the removal of most of the excess hydrochloric acid and adjustment of the pH to 1.5, mercuric acetate was added; in most cases this precipitated the amino acids, but not the Cm-amino acids. These were then precipitated with mercuric nitrate.

Glycylglycine—The peptide, prepared by the method of Dunn, Butler, and Deakers (21), was carboxymethylated with 9 molar equivalents of bromoacetate for 3 days at 36°. After filtration to remove excess MgO, 5 volumes of ethanol were added. The amorphous product was redissolved in a small quantity of water and reprecipitated by adding 5 volumes of ethanol. It contained 7.0 per cent of N but was not further characterized.

A 150 mg. portion (0.75 m.eq. of N) was hydrolyzed in 4 ml. of 6 N HCl for 22 hours under a reflux. The solution was evaporated under reduced pressure, most of the excess of HCl being removed by repeated vacuum distillation after addition of water. The residue was then treated with 5 ml. of water, when di-Cm-glycine immediately crystallized. After 20 hours at 0–5° the product was collected, washed with cold water, and dried. The yield was 65 mg. (91 per cent of the theoretical amount) of di-Cm-glycine. It melted with decomposition at 242–251°.

$C_6H_9O_6N$. Calculated, N 7.3; found, N 7.3

The filtrate from the crude di-Cm-glycine was made alkaline, shaken with benzoyl chloride, and acidified. The precipitate, after being washed with ligroin and recrystallized, yielded 45 mg. of hippuric acid, m.p. 183° (64 per cent of the theoretical amount).

Leucylglycylglycine—The peptide, synthesized by the method of Schott, Larkin, Rockland, and Dunn (22), possessed the melting point and specific rotation recorded by these authors. A 0.75 mmole portion was carboxymethylated by the standard procedure. The filtrate from the excess MgO was acidified to Congo red and treated with an excess of saturated mercuric nitrate. The resulting precipitate was well washed with water and decom-

posed with H_2S. The filtrate was concentrated *in vacuo* to 2 ml., made alkaline with calcium hydroxide, and treated with 7 volumes of ethanol. The precipitate was washed with ethanol, redissolved in water, filtered, reprecipitated with ethanol, and dried over P_2O_5 at room temperature. The yield was 317 mg. of a calcium salt containing 1.99 m.eq. of N (88 per cent of the theoretical amount).

A 307 mg. portion of this product (1.92 m.eq. of N) was hydrolyzed in 6 ml. of 6 N HCl under a reflux for 40 hours. After removal of excess HCl under reduced pressure, the residue was dissolved in 3 ml. of water and the solution, which gave no precipitate with mercuric acetate, was treated with saturated mercuric nitrate. The precipitate was well washed with water, dried (347 mg.), dissolved in 15 ml. of M NaCl, and reprecipitated with mercuric nitrate. After being washed and dried over $CaCl_2$, the product contained 0.55 m.eq. of N (86 per cent of the theoretical amount). It melted at 169–171° with decomposition.

$C_{20}H_{28}O_{12}N_2Hg_3$. Calculated, N 2.6, Hg 55.2; found, N 2.2, Hg 56.1

Glutathione—A solution of 0.55 mmole of the tripeptide in 10 ml. of water was treated with MgO, aerated until it no longer reduced phosphotungstic acid, and carboxymethylated with 6 mmoles of bromoacetate. The filtrate was acidified with concentrated HCl (0.2 ml.) and treated with saturated mercuric nitrate (11 ml.). The well washed precipitate was decomposed with H_2S. The filtrate was made alkaline with calcium hydroxide and twice precipitated with ethanol. It weighed 254 mg. and contained 1.30 m.eq. of N (79 per cent of the theoretical amount).

A sample of the calcium salt containing 0.586 m.eq. of N was hydrolyzed in 3 ml. of 6 N HCl under a reflux for 24 hours; the excess of HCl was evaporated and the residue dissolved in 2.5 ml. of water. The solution was adjusted to pH 1 and treated with 2 ml. of 1 M mercuric acetate; the precipitate, presumably the mercuric complex of cystine, weighed 109 mg. and contained 0.154 m.eq. of N (79 per cent of the theoretical amount).

On addition of 3 ml. of saturated mercuric nitrate to the supernatant solution and washings, the mercury derivative of the di-Cm-glutamic acid was collected. After reprecipitation, this weighed 99 mg. and contained 0.144 m.eq. of N (74 per cent of the theoretical amount). It melted with decomposition at 174–175°.

$C_9H_9O_8NHg_2$. Calculated, N 2.1, Hg 60.7; found, N 2.0, Hg 58.8

Alanyllysylalanine—An aqueous solution (kindly furnished by the late Dr. Erwin Brand of this laboratory) of 0.43 mmole of the peptide (23) was carboxymethylated with 4.3 mmoles of bromoacetate; the mercuric derivative of the product was prepared and decomposed with H_2S. The car-

boxymethylated tripeptide, precipitated as its calcium salt with the aid of ethanol, weighed 161 mg. and contained 0.96 m.eq. of N (56 per cent of the theoretical amount).

A solution of 149 mg. of this calcium salt, containing 0.89 m.eq. of N, in 3 ml. of 6 N HCl was boiled under a reflux for 18 hours. The excess of HCl was removed under reduced pressure and the residue dissolved in 3 ml. of water. To this solution, mercuric acetate solution (1 molar in 1 per cent acetic acid) was added until precipitation was complete (2 ml.). The supernatant solution and washings were concentrated *in vacuo* to 2 ml. and a second small quantity of precipitate was collected. There were thus obtained 224 mg. of a mercury salt of ϵ-di-Cm-lysine containing 0.44 m.eq. of N (99 per cent of the theoretical amount). The compound was purified by solution in dilute sodium chloride and reprecipitation with mercuric acetate. It melted with decomposition at 165–167°.

$C_{10}H_{18}O_6N_2ClHg_2$. Calculated. N 4.0, Cl 5.1, Hg 57.6
Found. " 4.0, " 5.4, " 56.2

The product obtained after removal of mercury failed to crystallize.

The supernatant solution from the mercuric ϵ-di-Cm-lysine was treated with 1.3 ml. of saturated mercuric nitrate, which precipitated 151 mg. of mercuric di-Cm-alanine containing 0.22 m.eq. of N (99 per cent of the theoretical amount). After two reprecipitations the product melted with decomposition at 177–178°.

$C_{14}H_{16}O_{12}N_2Hg_3$. Calculated, N 2.8, Hg 59.8; found, N 2.7, Hg 56.2

Glycylhistidine—Samples of this peptide (24), kindly provided by Dr. du Vigneaud, were carboxymethylated with 12 equivalents of bromoacetate under the standard conditions for 6 and for 25 hours. The Pauly reaction then indicated that 66 and 92 per cent, respectively, of the imidazole group had reacted. The reaction mixture from the 25 hour sample was acidified and treated with mercuric nitrate. The precipitate was washed and decomposed with hydrogen sulfide; the filtrate was rendered alkaline with calcium hydroxide, filtered, and treated with 2 volumes of ethanol. The precipitate contained 76 per cent of the nitrogen in the starting material.

A portion containing 0.54 m.eq. of N was hydrolyzed with boiling 6 N HCl for 20 hours. The excess of HCl was removed by distillation under reduced pressure, and the residue was dissolved in 3 to 4 ml. of water and adjusted to pH 2 with sodium hydroxide, when di-Cm-glycine rapidly crystallized. After a few days in the refrigerator the crystals were collected, washed with water, then with alcohol, and dried; yield, 19 mg.; m.p. 245–247° (decomposition).

$C_6H_9O_6N$. Calculated, N 7.3; found, N 7.4

The mother liquor was treated with mercuric acetate and an equal volume of ethanol, whereby a mercuric salt of imidazole-di-Cm-histidine was precipitated. After being washed with 50 per cent ethanol and dried, it weighed 119 mg.; m.p. 256–257° (decomposition).

$C_{10}H_9O_6N_3Cl_2Hg_3$. Calculated. N 4.5, Cl 7.6, Hg 64.0
Found. " 4.8, " 7.9, " 66.4

The filtrate was treated with mercuric nitrate, which precipitated the mercuric salt of a little di-Cm-glycine which had not crystallized. This product after reprecipitation weighed 15 mg.; m.p. 180–183° (decomposition).

$C_{12}H_{12}O_{12}N_2Hg_3$. Calculated, N 2.9; found, N 3.2

The total recoveries of di-Cm-glycine and di-Cm-histidine were accordingly 100 and 101 per cent of the theoretical amounts, respectively, on the basis of nitrogen content.

The 6 hour run yielded products with identical properties, but only 52 per cent of the peptide nitrogen was obtained as the Ca salt, from which the hydrolytic products were obtained in respective yields of 98 and 90 per cent. In this case, a small amount of precipitate appeared on the addition of mercuric acetate prior to the addition of alcohol. This was probably the imidazole mono-Cm- derivative but was not investigated.

Alanylhistidine—This peptide, synthesized by Dr. J. Polatnick of this laboratory by the method of Hunt and du Vigneaud (25), was employed in the form of its dihydrochloride, 450 mg. (1.5 mmoles) of which were carboxymethylated with 18 mmoles of bromoacetate for 24 hours at 35–37°. The product was precipitated with mercuric nitrate, regenerated with hydrogen sulfide, and isolated as the alcohol-insoluble calcium salt. This weighed 470 mg. and contained 2.85 m.eq. of N, corresponding to a yield of 48 per cent of the theoretical amount.

Hydrolysis of 213 mg. of this (1.29 m.eq. of N) was carried out in 5 ml. of boiling 6 N HCl. The excess of acid was volatilized; the residue was dissolved in water and the pH raised from 0.3 to 1.6 in a final volume of 5 ml. No precipitate was formed on the addition of mercuric acetate.[2]

Mercuric nitrate gave 425 mg. of a precipitate which contained 1.18 m.eq. of N (91 per cent of total) and was therefore a mixture. After reprecipitation of 406 mg., the weight fell to 298 mg.; the product still contained three times as much nitrogen (0.95 m.eq.) as could have been present as di-Cm-alanine. A 282 mg. portion (0.90 m.eq. of N) was decomposed

[2] When this experiment was performed, it had not yet been recognized that precipitation of mercuric imidazole-di-Cm-histidine could be elicited by addition of alcohol.

with H_2S; the filtrate was concentrated to 13 ml. and shaken with five 100 ml. portions of ethyl acetate. The evaporated extract was dissolved in 3 ml. of ethanol. By addition of 8 ml. of ethyl acetate, followed by 30 ml. of light petroleum, 15 mg. of pure di-Cm-alanine (m.p. 207–208°; N, 6.8) were secured.

The aqueous solution remaining after the extraction with ethyl acetate was diluted with 3 volumes of isopropanol and chilled overnight in the refrigerator. The solid which separated contained 0.64 m.eq. (95 per cent of the theoretical amount) of N. After two recrystallizations from water-isopropanol it melted at 263–264° (decomposition) and gave analytical values for imidazole-di-Cm-histidine.

$C_{10}H_{13}O_6N_3$.	Calculated.	C 44.3, H 4.8, N 15.5, NH_2-N 5.2
	Found.	" 44.3, " 5.0, " 15.5, " 5.1

On treatment with mercuric nitrate, this compound yielded a precipitate which melted with decomposition at 203° after darkening at 196–198°. Mercuric acetate yielded a precipitate (m.p. 256–257°, decomposition) in 50 per cent alcoholic, but not in aqueous, solution.

Chromatography of Cm-amino Acids—The solvent employed was a mixture of *n*-butanol (2 volumes), ethanol (1 volume of 95 per cent), and aqueous ammonia (2 volumes of 3 N). The paper was No. 1 Whatman, 15 to 20 cm. in width. After at least 4 hours for equilibration with the atmosphere within the vessel, upward chromatographic migration was allowed to proceed for about 20 hours at room temperature (29–31°). The solvent front was marked; the paper was dried at 70–80° for 15 to 20 minutes, sprayed with the reduced ninhydrin reagent of Long, Quayle, and Stedman (19), heated at 110–115° for 1 to 3 minutes, and allowed to stand at room temperature overnight. The background color had then faded; the spots of di-Cm-amino acids were purple, those of mono-Cm-amino acids pink, and those of native amino acids dark bluish brown.

Distribution Coefficients—Solutions of about 20 mg. of each Cm-amino acid in 3 ml. of 0.1 N HCl were shaken for 90 minutes at room temperature with appropriate volumes (7 ml. or 12 ml.) of ethyl ether or ethyl acetate. When the layers had separated, measured samples of each were withdrawn and subjected to Kjeldahl analysis. The results are presented in Table V.

SUMMARY

1. Simple α-amino acids react with bromoacetate at pH 9, with replacement of the amino hydrogen atoms by carboxymethyl groups. In most cases, two such groups enter the molecule, but monosubstitution products can be isolated from phenylalanine and glutamic acid, and with aspartic acid the second group enters hardly at all.

2. Under the conditions adopted, the hydroxyl group of tyrosine, the ϵ-amino group of lysine, and the imidazole group of histidine also accept carboxymethyl groups. The guanidino group of arginine does not react.

3. Carboxymethylamino acids form insoluble mercuric salts on treatment with mercuric nitrate, but not with mercuric acetate.

4. On treatment with bromoacetate, peptides form products from which, after acid hydrolysis, the carboxymethylated *N*-terminal amino acids can be isolated in high yields.

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CARBOXYMETHYL PROTEINS*

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The action of iodoacetate on proteins has formed the subject of many studies, in most of which attention has been primarily directed to the reaction with SH groups. Although the possibility that other groups may be involved has been considered (1), little information is available.

In the experiments here reported, proteins have been subjected to the action of bromoacetate at the pH of about 9 maintained by an aqueous suspension of magnesium oxide. Most of these studies have been carried out with crystalline bovine serum albumin, which yielded products insoluble at pH 2 but soluble at pH 5. Preliminary experiments on a few other proteins have indicated that the reaction is generally applicable to soluble proteins, and that sodium or potassium carbonate may be employed in place of magnesia. The products from egg albumin and casein exhibit solubility behavior similar to that of carboxymethylated serum albumin, as does that from hemoglobin, from which the porphyrin is removed during the reaction. Gelatin yields products which contain no amino groups and give no color with ninhydrin, but display essentially unaltered gelling characteristics. On the other hand, zein, wool, and silk do not dissolve when treated with bromoacetate in potassium carbonate solution.

Under the conditions employed, over 90 per cent of the amino groups of serum albumin react with bromoacetate in 16 hours, and amino nitrogen is no longer detectable after 24 hours.

Huggins and Jensen (1) have shown that treatment of serum proteins with iodoacetate results in loss of heat coagulability, an effect which they ascribed to the mutual repulsion of the negatively charged groups introduced. Similarly, the products of the action of bromoacetate on the albumins are not coagulated when heated in solution at pH 5 or above. The effect of carboxymethylation resembles denaturation in other respects. For example, as the reaction progresses, the disulfide linkages become increasingly accessible to the action of sulfite (Table I); this effect is not produced by MgO alone.

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XUM

As with histidine and tyrosine (2), the imidazole and phenolic groups of serum albumin react with bromoacetate to form derivatives which yield no color with diazobenzenesulfonic acid. In the native albumin, not all of these groups react with the Pauly reagent, for the color intensity developed with the protein is only 75 per cent of that observed[1] after hydrolysis (Table II). The disappearance of this discrepancy as the reaction with bromoacetate proceeds also points to the denatured character of the carboxymeth-

TABLE I

Reactive Disulfide Groups in Bovine Serum Albumin Made Available during Carboxymethylation

Reaction time	μeq. SS per m.eq. total N	
	Before hydrolysis	After hydrolysis
hrs.		
0	5.3	24
5	7.3	24
24	11.0	24
48	13.2	22
66	15.0	23

TABLE II

Disappearance of Diazo-Coupling Groups during Carboxymethylation of Bovine Serum Albumin

The values are expressed as micromoles of histidine per milliequivalent of total N.

Reaction time	Diazo-coupling groups	
	Before hydrolysis	After hydrolysis
hrs.		
0	22.3	29.8
5	20.3	26.0
24	15.7	18.8
48	12.6	12.9
66	11.3	9.1

[1] The values reported by Stein and Moore (3) indicate that bovine serum albumin contains 22.5 μmoles of histidine and 24.4 μmoles of tyrosine per milliequivalent of total N. We find the color intensity developed from tyrosine to be 29 per cent of that from an equimolar amount of histidine. From these values it can be calculated that the total color per milliequivalent of protein N should correspond to that which can be developed from 29.6 μmoles of histidine, in close agreement with the observed value of 29.8.

ylated protein. On the other hand, the complete carboxymethylation of the phenolic groups in a sample of bovine albumin which had been subjected to the action of bromoacetate for 64 hours is suggested by its ultraviolet absorption curves (Fig. 1) which, in contrast to those for the original protein, do not show the shift of maximum with alkali characteristic of proteins

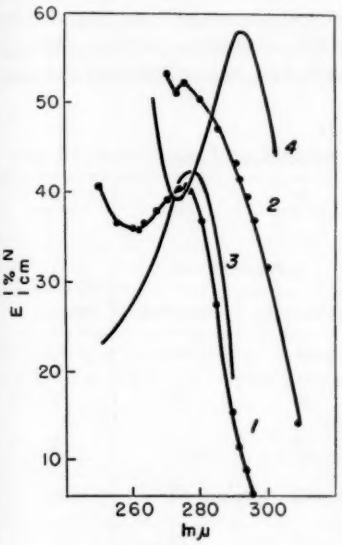

FIG. 1

FIG. 2

FIG. 1. Absorption spectra of native and carboxymethylated bovine serum albumin. Curve 1, Cm-albumin at pH 6.6; Curve 2, Cm-albumin in 0.1 N NaOH; Curve 3, native albumin at pH 4.5; and Curve 4, native albumin in 0.1 N NaOH.

FIG. 2. Ultracentrifugal sedimentation patterns. *A*, carboxymethylated bovine serum albumin; *B*, crystalline bovine serum albumin; *C*, serum albumin treated with MgO; *D*, carboxymethylated serum albumin recovered from urine.

containing combined tyrosine (4). In acid solution, the albumin and its carboxymethyl (Cm-) derivative[2] display nearly coincident absorption maxima (275 to 280 mμ) and extinction coefficients based on nitrogen content.

From the sedimentation patterns of bovine serum albumin (Fig. 2, *B* and *C*) before and after treatment with magnesium oxide alone, it appears that no appreciable change in the particle size of the protein is brought about by the exposure to magnesia. The sedimentation constant of the

[2] Hereafter referred to as Cm-.

crystalline protein (*B*) is 3.2 S units at 15°, and this, when corrected for temperature, density, and viscosity, gives $s_{20°} = 4.0$. The control sample (*C*) which had been stirred with a suspension of MgO for 72 hours at 37° showed 3.5 S units at 26° and $s_{20°} = 3.7$. Both samples gave evidence of the presence of a second, faster moving component possibly because of the presence of a trace of a bivalent metal. The carboxymethylated sample (*A*) appeared to be homogeneous, and lacked the second component. Its sedimentation constant was 1.2 S units at 26°, whence $s_{20°} = 2.0$. Approximate values for the apparent diffusion coefficient, calculated from the same

TABLE III

Action of Trypsin on Progressively Carboxymethylated Bovine Serum Albumin

Substrates (1.0 m.eq. N in each)		A	B	C	D
Carboxymethylation		0 hr.	4.5 hrs.	8 hrs.	28 hrs.
NH_2 content, *μeq.*		86	49	15	0
Time	Trypsin added	Total NH_2 liberated			
		Substrate A	Substrate B	Substrate C	Substrate D
hrs.	*γ N*	*μeq.*	*μeq.*	*μeq.*	*μeq.*
0	81				
3		67	52	24	21
6	27				
23		76	67	55	26
25	70				
28		88	73	67	29
29	54				
45		88	79	67	28
46	83				
49		100	91	67	34

data by the method of Svedberg and Pedersen (5), were 4.3×10^{-7} cm.² per second for the control bovine albumin and 1.0×10^{-7} cm.² per second for the carboxymethyl derivative. These values, taken in conjunction, indicate that no notable degradation of the protein occurs during the carboxymethylation.

Introduction of the carboxymethyl groups markedly reduces the susceptibility of bovine albumin to the action of trypsin, the degree of inhibition being dependent on the time of treatment with bromoacetate (Table III). Conversely, carboxymethylation of trypsin caused loss of enzymatic activity (Table IV), which decreased to one-third after 3 hours and to one-twentieth after 7.5 hours of treatment with bromoacetate. This diminution of activity was roughly proportional to the progressive decrease of amino nitrogen on carboxymethylation. Treatment with magnesia suspen-

sion alone had little effect on the proteolytic activity of the dialyzed product, although extensive autolysis occurred.

Carboxymethylated bovine serum albumin, when injected in neutral solution either intravenously or intraperitoneally into rats, was rapidly and extensively excreted in the urine, 16 to 19 per cent of the dose being recoverable in 24 hours (Table V). The excreted Cm-albumin appeared, from its sedimentation pattern (Fig. 2, *D*), to have undergone no appreciable degradation. Similar results, not here described, were obtained with carboxymethylated casein and globin.

TABLE IV

Enzymatic Activity of Carboxymethylated Trypsin

	Crystalline trypsin	Control	3 hr. carboxymethylation	7.5 hr. carboxymethylation
EN*	0.91	0.76	1.58	3.39
NH_2-EN† (2 hrs.)	0.80	0.70	0.23	0.04
" (19 hrs.)	1.24	1.25	0.50	0.11

* N content (mg.) of enzyme preparation taken.

† Ratio of NH_2-N set free to N in enzyme taken.

TABLE V

Urinary Excretion of Cm-Bovine Albumin

Rat No.	Cm-protein N injected	Method of injection	Cm-protein N recovered
	mg.		*mg.*
1	38.4	Intravenous	6.1
2	15.0	Intraperitoneal	2.9
3	26.8	"	4.4

The passage of Cm-proteins through the kidney is possibly referable to their preponderantly anionic character. It cannot be ascribed to tissue damage, for the urine samples from which Cm-albumin had been precipitated by acidification gave no precipitate on the addition of trichloroacetic acid.[3] Moreover, pathological examination[4] of the liver and kidneys of the experimental animals revealed no evident lesion.

A preliminary experiment on the application of the procedures outlined in the foregoing report (2) to a hydrolysate of the Cm-bovine albumin indicated the possible utility of this type of derivative for the identification

[3] Control tests indicated that less than 2 mg. of serum proteins in a 24 hour sample of urine can be detected by this procedure.

[4] Kindly performed by Dr. Abner Wolf of this College.

and estimation of terminal α-amino groups in proteins. After the removal of products precipitable by mercuric acetate, addition of mercuric nitrate caused the precipitation of a solid which showed the properties of mercuric Cm-aspartate. The amount corresponded approximately to that expected for one terminal aspartyl group (6) in a protein of molecular weight 69,000, rather than to the larger and more diversified number of groups recently reported (7).

EXPERIMENTAL

Carboxymethylation of Bovine Serum Albumin

An aqueous solution containing 500 mg. of the crystalline protein (12 per cent moisture), 6 mmoles of bromoacetate, and excess of MgO in 20 ml. was gently agitated at 35°. Filtered samples were periodically removed. Measured volumes of these were analyzed for amino nitrogen, for diazo coupling (imidazole plus phenolic), and for disulfide groups. In the diazo tests, 0.2 ml. samples were diluted with 5 ml. of water and treated successively with 1 ml. of 1 per cent diazobenzenesulfonic acid; after 5 minutes, 3 ml. of 20 per cent Na_2CO_3 and 10 ml. of 20 per cent ethanol were added. The mixtures were diluted to 25 ml. and the color intensity was measured photoelectrically with the use of a green filter. For the estimation of disulfide groups, 0.5 ml. samples were treated with 2 ml. of the phosphotungstic acid solution of Folin (8), 10 ml. of 0.1 N NaOH, and 1 ml. of 1 M Na_2SO_3. The mixtures were allowed to stand for an hour at 25° and the color was measured with the use of a red filter. No color was developed when the addition of the sulfite was omitted. These tests were also carried out on neutralized acid hydrolysates of the analytical samples.

After 72 hours, the remainder of the reaction mixture was centrifuged; the supernatant solution was acidified to pH 2 with HCl, cooled for several hours in the refrigerator, and centrifuged. The N content of the supernatant liquid showed that 99.6 per cent of the Cm-protein had been precipitated by the HCl. The product redissolved when the pH of a suspension was raised to 5, and could again be completely precipitated at pH 2. When a dry product was required, the solid was repeatedly washed with water, then with alcohol, and dried *in vacuo* over calcium chloride at room temperature. A typical preparation was found to contain N 13.7 and S 1.76 per cent.

Action of Trypsin on Cm-albumin

The substrates referred to in Table III were prepared for the periods indicated by the procedure outlined above. The control sample (Substrate A) was treated with magnesia alone for 25 hours. The reaction mixtures were centrifuged; the clear solutions were adjusted to pH 7 with dilute HCl

and dialyzed at 5°. After determination of total and amino (Van Slyke) nitrogen, quantities containing 1.0 m.eq. of N were diluted to 5 ml. and mixed with 5 ml. of 0.2 M phosphate buffer, pH 8. The resulting mixtures were treated with a solution of crystalline trypsin in the quantities and at the times shown. Aliquots were periodically withdrawn for formol titration. The results represent the increases in NH_2-N.

Carboxymethylation of Trypsin

Twice recrystallized trypsin containing 8.4 per cent total N and about 50 per cent of magnesium sulfate was carboxymethylated in 0.1 gm. quantities with 0.6 mmole of bromoacetate in a total volume of 2 ml. for 3 and 7.5 hours. A control was similarly carried out for 7.5 hours with MgO alone. In each case the excess MgO was removed and the solution neutralized and dialyzed at 5°. In the control sample, autolysis appeared to have been extensive, for on dialysis more than 70 per cent of the nitrogen passed through the bag, and, of the nitrogen remaining in it, 20 per cent was present as NH_2-N. In the presence of bromoacetate, much less autolysis occurred: after 3 hours, 53 per cent was dialyzable and the ratio of amino to total nitrogen was 0.05; after 7.5 hours the corresponding values were 29 per cent and 0.02.

Appropriate amounts of the three preparations were added to solutions of 0.1 gm. of crystalline bovine albumin in 0.1 M phosphate buffer, pH 8, to give a total volume of 10 ml. Aliquot portions were taken for formol titration after 2 and 19 hours digestion at 37°. The values in ratio of liberated NH_2-N to enzyme N are presented in Table IV.

Urinary Excretion of Cm-bovine Albumin

The Cm-protein (72 hours treatment), twice precipitated by acid and thoroughly washed with water, was suspended in 10 parts of water and dissolved by addition of $NaHCO_3$ to pH 7. Solutions thus prepared were injected intravenously or intraperitoneally into rats weighing 200 to 250 gm. The urine collected during the ensuing 24 hour period was clarified by centrifugation and acidified to pH 2.5 with HCl. The precipitate was centrifuged, washed twice with water, redissolved at pH 5, reprecipitated with acid, well washed, and redissolved. The amounts of Cm-protein in the solutions injected and recovered were estimated by determination of N in aliquot portions. The results are shown in Table V. In a control experiment, in which a solution of 334 mg. of crystalline bovine albumin in 3.8 ml. was injected intravenously into a rat, the urine yielded no precipitate with trichloroacetic acid.

The Cm-albumin isolated from the urine of Rat 3 was dialyzed against 0.1 M phosphate buffer of pH 6.6 and its sedimentation pattern determined in the ultracentrifuge (Fig. 2, *D*).

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Ultracentrifugal Sedimentation

The carboxymethylated albumin (Fig. 2, *A*), prepared by the usual procedure (see above) during 72 hours, the crystalline albumin (Fig. 2, *B*) and its control (Fig. 2, *C*), treated with a suspension of magnesium oxide for 72 hours at 35°, were dialyzed against phosphate buffer, pH 6.6. All solutions were finally adjusted to a concentration of 1 per cent of protein for the sedimentation experiments.

Terminal α-Amino Group in Bovine Albumin

A sample of the protein, carboxymethylated for 27 hours, was boiled under a reflux with 6 N HCl for 20 hours, and a portion of the clear hydrolysate containing 15.0 m.eq. of N was evaporated under reduced pressure. The excess of HCl was removed by repeated distillation under reduced pressure, with addition of water and, finally, of toluene. The residue was dissolved in 10 ml. of water and treated with 20 ml. of 1 M mercuric acetate solution. The precipitate was centrifuged and washed. The united supernatant solution was treated with an equal volume of ethanol, which brought down a further small quantity of precipitate; this was centrifuged and washed with 1:1 aqueous alcohol. Saturated mercuric nitrate in excess was added to the combined solution, and the resultant precipitate was centrifuged, dissolved in 0.4 M NaCl, reprecipitated with mercuric nitrate, washed with water, and dried. It weighed 8.0 mg.

This substance showed the same melting point (179–182°, decomposition) and chromatographic behavior (R_F 0.34) as mercuric Cm-aspartate; with the reduced ninhydrin reagent, the chromatographic spot developed the pinkish purple color characteristic of mono-Cm-amino acids (2). The remainder of the mercuric salt was decomposed with H_2S; the filtrate gave no color with ninhydrin under the usual conditions.

The amount of nitrogen (0.23 mg.) in the above product, computed on the assumption that it consisted solely of mercuric Cm-aspartate, corresponds to 0.11 per cent of the total N in the hydrolysate. The theoretical value is 0.126 per cent for one terminal α-amino group in bovine albumin containing 16.07 per cent of N (3) and with a molecular weight of 69,000.

SUMMARY

1. Bovine serum albumin reacts with bromoacetate at pH 9 to yield derivatives in which the entrant carboxymethyl groups are attached to the amino, phenolic, and imidazole groups.

2. No apparent degradation of the protein occurs in the reaction with bromoacetate.

3. The carboxymethylated protein is insoluble in water at pH 2, but soluble at pH 5 and above. It is not coagulable by heat and resembles a denatured protein in the increased chemical accessibility of its disulfide and imidazole groups.

4. The carboxymethylated protein, when administered intravenously or intraperitoneally to rats, is rapidly excreted through the kidney in essentially unchanged condition.

5. Carboxymethylation offers promise as a means for the estimation and identification of terminal α-amino groups in proteins.

The authors are deeply indebted to Dr. Irwin B. Wilson and Dr. E. Ellenbogen for advice and supervision in the ultracentrifugal studies, and to Dr. Abner Wolf for the pathological examinations.

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PEPTIDES OBTAINED BY PEPTIC HYDROLYSIS OF PERFORMIC ACID-OXIDIZED RIBONUCLEASE

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In a continuation of the structural studies on ribonuclease reported in earlier papers (1–3), the products resulting from the action of pepsin on performic acid-oxidized ribonuclease have been examined by means of ion exchange chromatography. Eight peptides have been isolated in purified form and their amino acid compositions have been determined. In a following communication (4), a partial structural formula for oxidized ribonuclease is proposed which is derived by a joint consideration of the peptides obtained after hydrolysis by pepsin, trypsin (3), and chymotrypsin (4).

EXPERIMENTAL

Materials—Oxidized ribonuclease was prepared in 200 mg. lots from Armour crystalline ribonuclease (Lot 381059) by the method previously described (3). The pepsin used was a twice recrystallized sample obtained from the Worthington Biochemical Corporation, Freehold, New Jersey (Lot PM538).

Hydrolysis of Oxidized Ribonuclease by Pepsin—Rates of hydrolysis were determined at 25° in a 0.2 M sodium citrate buffer at pH 1.9 and at pH 2.2. The buffer at pH 2.2 was prepared from 105 gm. of citric acid, 42 gm. of sodium hydroxide, and 80 ml. of concentrated hydrochloric acid made up to 5 liters; concentrated hydrochloric acid was added to bring the buffer to pH 1.9. The course of hydrolysis at an enzyme concentration of 0.02 per cent and at substrate concentrations of 1 and 4 per cent was followed by the ninhydrin procedure (5). Samples to be chromatographed were brought to pH 7 with 2 N NaOH to terminate the enzymatic action. After an interval of about 30 minutes, the sample was brought to pH 2.2 before it was transferred to the column.

Chromatography of Peptides on Columns of Dowex 50-X2—The resin used in this work was Dowex 50-X2, Lot 3328-12 (200 to 400 mesh), purchased from The Dow Chemical Company, Midland, Michigan. The method of preparing and operating the Dowex 50-X2 columns has been described in detail in a previous communication (3). Peptic hydrolysates from 40 mg. of oxidized ribonuclease were analyzed on 150 × 0.9 cm. columns. Larger columns (150 × 1.8 cm.) were used for the fractionation of hydrolysates of 200 mg. of oxidized ribonuclease. The N and 2 N sodium acetate-cit-

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rate buffers at pH 5.1 were added to the upper reservoir of the mixing device at effluent volumes of 1256 and 2750 ml., respectively (*cf.* Fig. 2). At the conclusion of the experiment, the resin was flushed from the inverted column with distilled water, transferred to a Büchner funnel, and washed with 0.2 N NaOH. The alkaline eluate (60 ml.), containing any ninhydrin-positive materials still held by the resin at pH 5, was promptly neutralized with 6 N HCl.

When columns 0.9 cm. in diameter were used, alternate effluent fractions were analyzed directly by the modified ninhydrin method (5), and the remaining fractions were analyzed after alkaline hydrolysis (3). With columns 1.8 cm. in diameter, aliquots were pipetted for analysis both with and without hydrolysis. Thus peptides giving a low color yield in the ninhydrin reaction were not overlooked. Quantitative amino acid analysis was carried out (3) on an aliquot of the pooled fractions containing each peptide. The aliquot was of a volume calculated to yield about 0.5 to 1 μmole of each constituent amino acid present as a single residue.

Peptides 9 and 10 were desalted before analysis by exchange of sodium for ammonium acetate on a column of ammonium Dowex 50-X2, after which the ammonium salt was removed by lyophilization (*cf.* (3)). Most of the sodium ion was removed from Peptides 5 and 7 prior to acid hydrolysis by concentrating the solution to dryness, extracting the residue with concentrated HCl, and removing the precipitated sodium chloride by centrifugation. Peptides 2, 3, 6, 9, 10, and 11 were hydrolyzed with 6 N HCl under reflux for 22 hours. Peptides 5 and 7 were hydrolyzed for 22 hours at 115° with 6 N HCl in evacuated sealed tubes.

Chromatography of Peptides on Columns of Amberlite IRC-50 (XE-64)—Since short times of peptic hydrolysis yielded appreciable percentages of peptides which could be eluted from Dowex 50 only by NaOH, a study was made of the possibility of fractionating these strongly bound segments on a carboxylic acid resin. Columns 30 × 0.9 cm. and 60 × 0.9 cm. were prepared with resin (XE-64, batch No. 2165, 200 to 400 mesh) that had been pretreated in the manner described for the chromatography of ribonuclease (6). To obtain the chromatogram shown in Fig. 3, a 4 hour peptic hydrolysate of oxidized ribonuclease (2 ml. volume) was adjusted to pH 5.85 and applied to a column 54 × 0.9 cm. equilibrated at pH 6.02 with 0.2 M phosphate buffer made up as described in the preceding studies (7). Effluent fractions of 1 ml. were collected from which 0.1 ml. aliquots were taken for analysis by the ninhydrin method.

Results

The curves shown in Fig. 1 give the rates of hydrolysis of oxidized ribonuclease by pepsin under two experimental conditions. Chromatographic

analysis was performed upon peptide mixtures obtained after 4 hours of hydrolysis at an enzyme-substrate ratio of 1:230 (similar to hydrolysate for Curve A, Fig. 1) and after 24 hours of hydrolysis at an enzyme-substrate ratio of 1:50 (similar to hydrolysate for Curve B, Fig. 1). From the increase in ninhydrin color, it was calculated that seven and eleven peptide bonds, respectively, could have been split quantitatively (twelve are hydrolyzed by trypsin (3)). In actuality, as will appear later, incomplete hydrolysis of a larger number of bonds undoubtedly takes place.

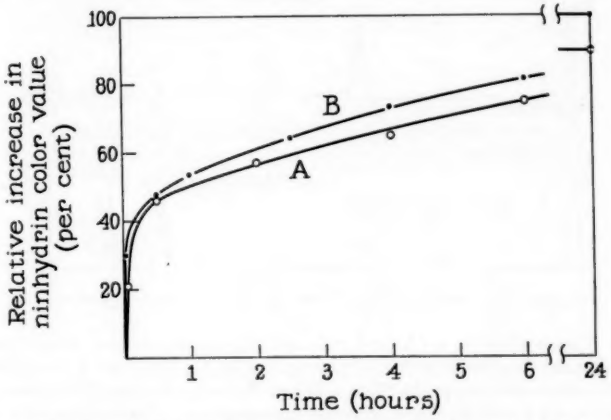

FIG. 1. The rate of hydrolysis of oxidized ribonuclease by pepsin at 25°. Curve A, pepsin concentration, 0.02 per cent; oxidized ribonuclease concentration, 4 per cent; pH 2.2. Curve B, pepsin concentration, 0.02 per cent; oxidized ribonuclease concentration, 1 per cent; pH 1.9. The curves have been corrected for the small amount of autodigestion occurring in control solutions of the enzyme.

Chromatography of the peptides obtained from 187 mg. of oxidized ribonuclease after a restricted treatment with pepsin (Curve A) gave the effluent curve shown by the solid line in Fig. 2. As indicated by the dash line, the peaks numbered 5, 6, 7, and 10 were found to rise when the enzyme-substrate ratio was increased to 1:50 (the pepsin concentration remaining constant) and the hydrolysis time was lengthened to 24 hours. The yield of Peptide 2 decreased under these conditions. The results obtained after hydrolysis of aliquots of each of the effluent fractions with alkali have not been included on the curve, inasmuch as no ninhydrin-negative peptides were found. The peak given by ammonia (accumulated from the reagents) was identified from its disappearance during alkaline hydrolysis.

The molar ratios summarized in Table I, considered together with the

chromatographic results (Fig. 2), indicated that the peaks labeled 2, 3, 5, 6, 7, 9, and 10 probably had arisen from individual peptides. The amino acid composition of each of the principal peptides is shown in Fig. 2 above the appropriate peak. Amino acid analyses of the fractions derived from the peaks labeled 1, 4, and 8 indicated that single peptides were clearly not

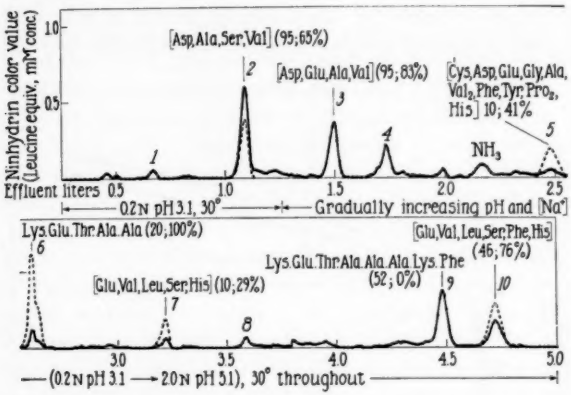

FIG. 2. The peptides in a 4 hour peptic hydrolysate of oxidized ribonuclease prepared under the conditions given for Curve A, Fig. 1. The dash line indicates the sizes of the corresponding peaks when a 24 hour hydrolysate prepared under the conditions given for Curve B, Fig. 1, was chromatographed. Chromatography of a hydrolysate from 187 mg. of protein was carried out on a 150 × 1.8 cm. column of Dowex 50-X2. The mixing chamber for the influent buffer had a volume of 2450 ml. The effluent was collected in 10 ml. fractions. Aliquots (0.5 ml.) were removed for analysis by the ninhydrin method. The figures in parentheses give the yield of each peptide under the conditions of hydrolysis shown for Curve A and Curve B (Fig. 1), respectively. The yields were derived from the amino acid analyses of the peptides. The sequence of the amino acid residues in brackets is undetermined.

present in these instances and no attempt was made to resolve these mixtures further.

When the less completely hydrolyzed sample (Curve A) was chromatographed on Dowex 50-X2, a considerable quantity of the ninhydrin-positive material applied to the column was found to be so strongly adsorbed that elution with alkali was required. An attempt was therefore made to fractionate the hydrolysate on a column of IRC-50 (54 × 0.9 cm.) equilibrated at pH 6.02, with the results shown in Fig. 3. Amino acid analyses suggested that the peak labeled 11 had arisen from a large peptide of 42 or 43 amino acid residues not found in the eluate from the Dowex 50 column at pH 5. The last component eluted from the IRC-50 column (at 58 ml.

in Fig. 3) had the same amino acid composition as Peak 9 (Fig. 2) and the two have been assumed to be identical.

In accordance with the terminology adopted in the previous communication (3), the series of peptides obtained by peptic hydrolysis of oxidized ribonuclease is referred to in Table I by numbers with the prefix O-Pep. The composition of the individual peptides in terms of the integral number of residues of the constituent amino acids per molecule is given in boldfaced type. All values for amino acids present at as much as 0.01 of a residue are included. Many of the peptides contain significantly greater

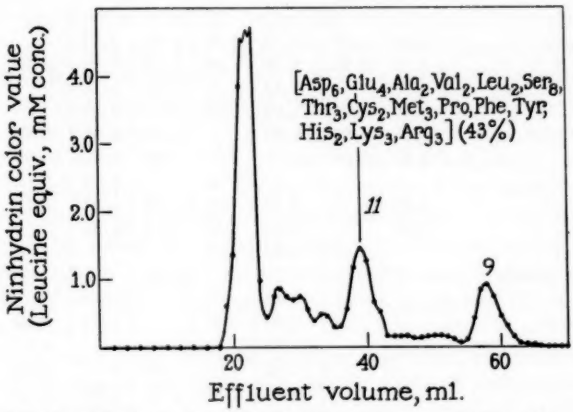

FIG. 3. The peptides in a 4 hour peptic hydrolysate of oxidized ribonuclease prepared under the conditions given for Curve A, Fig. 1. Chromatography of a hydrolysate from 40 mg. of protein was carried out on a 54 × 0.9 cm. column of IRC-50 (XE-64). A 0.2 M sodium phosphate buffer at pH 6.02 was used as eluent.

amounts of impurities than did the products obtained from the tryptic hydrolysates (3). The less specific character of the peptic hydrolysis apparently produces a larger number of peptides in low yield and the resulting mixture is fractionated less effectively by the column. Values for amide ammonia were not sufficiently accurate to warrant inclusion in Table I.

Tentative formulae have been assigned to O-Pep 9 and O-Pep 6 (*cf.* Fig. 2). The distinctive empirical formula of O-Pep 9, Glu,Thr,Ala_3,Phe,Lys_2, indicates that it arises from the amino-terminal end of the ribonuclease molecule (3) and corresponds to the previously isolated terminal heptapeptide, O-Tryp 10, plus a phenylalanine residue. This conclusion is given additional support by the isolation of the same octapeptide (O-Chy 25) from chymotryptic digests of oxidized ribonuclease (4). The structure Lys.Glu.Thr.Ala.Ala- assigned to O-Pep 6 is logical, assuming this peptide

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TABLE I

Amino Acid Composition of Peptide Fractions Obtained from Peptic Hydrolysis of Oxidized Ribonuclease

The amino acid composition of each peptide fraction obtained from chromatograms of the type shown in Figs. 2 and 3 is expressed in terms of the molar ratios of the constituent amino acids. The values for the principal components are given in bold-faced type. Values for amino acids present to less than 0.01 of a single residue are omitted.

Amino acid	Peptide							
	O-Pep 2	O-Pep 3	O-Pep 5	O-Pep 6	O-Pep 7	O-Pep 9	O-Pep 10	O-Pep 11
Aspartic acid	**1.04**	**1.01**	**1.05**	0.15	0.13	0.05		**5.76**
Glutamic "	0.16	**0.92**	**1.08**	**1.06**	**1.14**	**0.98**	**1.03**	**3.96**
Glycine	0.06		**1.14**	0.16	0.11	0.14		0.23
Alanine	**0.99**	**1.03**	**1.01**	**1.97**	0.12	**3.19**		**2.16**
Valine	**0.97**	**1.03**	**1.77**		**0.93**	0.07	**0.94**	**2.09**
Leucine	0.04	0.01		0.11	**1.00**	0.05	**0.98**	**2.06**
Isoleucine				0.06		0.02	0.04	0.07
Serine	**0.95**	0.12	0.33	0.22	**1.18**	0.24	**1.09**	**7.31***
Threonine	0.06		0.23	**1.12**	0.08	**0.96**		**3.12**
Cysteic acid			**1.20**	0.05				**2.21**
Methionine sulfone					0.05			**2.52**
Proline			**1.88**					**1.18**
Phenylalanine			**0.92**	0.11		**0.86**	**0.99**	**1.13**
Tyrosine	0.06		**0.92**†	0.06			0.05	**1.04**
Histidine			**0.93**		**0.94**	0.11	**1.06**	**2.16**
Lysine	0.09		0.17	**0.97**	0.07	**1.97**		**3.09**
Arginine								**2.89**
Total No. of residues	4	4	12	5	5	8	6	42–43

* An approximate correction of 10 per cent for decomposition of serine during hydrolysis makes the molar ratio of serine doubtful in this instance. A value of 8 rather than 7 residues has been included in Fig. 2 and appears more likely to be the correct value on the basis of the formula given in the accompanying paper (4).

† Low recoveries of tyrosine have been observed when small quantities of tyrosine-containing peptides are hydrolyzed in the presence of large amounts of acetate or citrate buffers, as noted in the accompanying paper on the chymotrypsin series of peptides (4). The above value for tyrosine is based upon the amount of the tyrosine in the hydrolysate (molar ratio of 0.57) plus the quantity of the tyrosine decomposition product emerging near the position of monochlorotyrosine in the chromatographic analysis.

to be derived from O-Pep 9 during the more extensive hydrolysis. This assumption is strengthened by the fact that O-Pep 9 could no longer be detected after the more drastic hydrolysis by pepsin, while the yield of O-Pep 6 rises concomitantly to 100 per cent.

O-Pep 2 (Asp,Ala,Ser,Val) has the same amino acid composition and chromatographic behavior as O-Chy 4 (*cf.* (4)), and hence the two may be assumed to be identical. The same peptide has been isolated from peptic hydrolysates of unoxidized ribonuclease by Anfinsen (8). In the accompanying paper (4) it is concluded that this valine-containing tetrapeptide occurs at the carboxyl-terminal end of the chain and that O-Pep 5, containing 12 residues, is the segment immediately preceding O-Pep 2 in the carboxyl-terminal sequence.

DISCUSSION

When the peptides isolated from peptic hydrolysates are considered in the light of the partial formula for oxidized ribonuclease proposed in the accompanying communication (4), it would appear that pepsin has acted predominantly at the two ends of the peptide chain. O-Pep 2 and 5 are derived from the last 16 residues in the carboxyl-terminal sequence, while O-Pep 3, 6, 7, 9, 10, and 11 all are split from the first 55 residues starting at the amino-terminal end. The central section of the chain has not been accounted for. The evidence from the present study for the individuality of O-Pep 11 is not strong. The likelihood that it represents a single peptide rests upon the fact that its amino acid composition fits a sequence of 43 amino acid residues following O-Pep 9 in the first half of the formula of the protein (4).

From the work with synthetic substrates (*cf.* (9) for a summary), pepsin would be expected to cleave oxidized ribonuclease preferentially at peptide bonds involving the 9 aromatic amino acid residues. It is clear from the present results, however, that a number of peptide bonds, in addition to those involving the aromatic amino acids, have been hydrolyzed and that not all of the peptides expected by splitting at tyrosine and phenylalanine residues have been formed. Moreover, several of the peptide fragments that have been identified are obtained in relatively poor yield. These facts indicate that the action of pepsin is rather complex, a conclusion that also emerges from the work of Sanger, Thompson, and Tuppy (10) on insulin and of Bell (11) on corticotropin. Pepsin is a less specific hydrolytic agent than trypsin or chymotrypsin for use in studies on the structure of proteins. Nevertheless, the peptides that have been isolated from peptic hydrolysates of oxidized ribonuclease have provided limited but useful information concerning the arrangement of amino acid residues in certain sections of the ribonuclease molecule.

SUMMARY

The mixture of peptides resulting from the hydrolysis of oxidized ribonuclease by pepsin has been fractionated on columns of Dowex 50-X2.

Eight peptides have been separated in purified form in yields of 10 to 100 per cent. About half of the 124 amino acid residues in the protein have been accounted for in terms of two tetra-, one hexa-, and one octapeptide and two large fragments of 12 and 43 amino acid residues, respectively. The results, although providing a less complete accounting for the amino acid residues in the molecule than experiments with trypsin and chymotrypsin, give evidence concerning the arrangement in both the amino-terminal and carboxyl-terminal sections of the peptide chain.

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PEPTIDES OBTAINED BY CHYMOTRYPTIC HYDROLYSIS OF PERFORMIC ACID-OXIDIZED RIBONUCLEASE. A PARTIAL STRUCTURAL FORMULA FOR THE OXIDIZED PROTEIN*

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In a previous investigation dealing with the structure of ribonuclease (2), trypsin was used as a specific hydrolytic reagent to cleave the single peptide chain of the performic acid-oxidized protein at the carboxyl groups of the lysine and arginine residues. Tryptic hydrolysis led to the formation, in good yield, of thirteen segments, which accounted for all of the molecule.[1]

The next stage in the structural study has been to establish the order in which the series of peptides formed by the action of trypsin is linked together in the original chain. The information required for the completion of this aspect of the study has been furnished by the present experiments in which oxidized ribonuclease is cleaved specifically by chymotrypsin at bonds other than those hydrolyzed by trypsin. The peptides formed have been separated on columns of Dowex 50-X2 and the amino acid composition of each has been determined quantitatively. From these data, coupled with the information derived from the series of peptides formed by the action of trypsin, it has been possible to deduce for the oxidized protein a partial structural formula that reveals the order in which the various peptides are linked to one another in the intact molecule. The additional information derived from the peptides liberated by the action of pepsin (3) has been incorporated in the formulation of the chain.

EXPERIMENTAL

Material—Performic acid-oxidized ribonuclease was prepared from Armour's crystalline ribonuclease (Lot 381059) by the procedure described previously (4). Each batch used in preparative scale work was subjected to quantitative amino acid analysis (4) prior to use to be certain that cys-

* A preliminary report of this work was presented at the Third International Congress of Biochemistry, Brussels, August 1–6, 1955 (1).

[1] As a working hypothesis it is assumed that ribonuclease has 4, not 5, proline residues and 15, rather than 16, aspartic acid residues, making a total of 124 residues per molecule (*cf.* under "Discussion" in Hirs *et al.* (2)).

tine had been converted to cysteic acid, methionine to the sulfone, and that all of the other amino acids, particularly tyrosine (4), were present in the expected quantities.

Crystalline, salt-free chymotrypsin, purchased from the Worthington Biochemical Corporation, Freehold, New Jersey (lots No. CD521 and CD-425SF), was used in this work. The trypsin content of these preparations was found to be 0.6 and 0.8 per cent, respectively, as determined by the rate of hydrolysis of carbobenzoxy-L-argininamide.

Hydrolysis of Oxidized Ribonuclease with Chymotrypsin—The rate of the chymotryptic hydrolysis of oxidized ribonuclease was determined at 25° exactly as described for the corresponding experiments with trypsin (2). The substrate was present at a concentration of 1.0 per cent in the phosphate buffer at pH 7.0, and the concentration of chymotrypsin was 0.005 per cent. The reaction was terminated by adding N HCl to bring the solution to pH 2.2.

Chromatography of Peptides on Columns of Dowex 50-X2—The primary separation of the peptides shown in Fig. 2 was effected on 150 × 1.8 cm. columns of Dowex 50-X2, operated in the Na form, by procedures identical to those described for the fractionation of the mixture of peptides obtained in the tryptic hydrolysis of oxidized ribonuclease (2). The eluents required for the rechromatography of the overlapping zones from the chromatogram shown in Fig. 2 were prepared by appropriate dilution of the stock 2 N sodium citrate-acetate buffer at pH 5.1 described in a previous paper (2).

The effluent fractions were collected and analyzed, and the amino acid analyses were performed in the manner described previously. All peptides were hydrolyzed for 22 hours at 110° with 6 N HCl in evacuated sealed tubes made from thick walled 50 ml. centrifuge bottles (Corning No. 8420). It has been noticed that, when peptides containing tyrosine are hydrolyzed in the presence of very high concentrations of acetate-citrate buffer, almost complete decomposition of tyrosine may occur, with the concomitant formation of about half the molar amount of a substance exhibiting the chromatographic behavior of chlorotyrosine (4). For this reason, the peptides present in Peaks 22 and 29 (Fig. 2), which require about a 1 M buffer for elution, were desalted (2) prior to the preparation of hydrolysates for the determination of tyrosine.[2]

[2] In current experiments in which Dowex 50-X2 columns are employed for the chromatography of peptides, sodium acetate buffers are being used in preference to sodium citrate buffers. If the salt concentration in the effluent is reduced by treatment of the aliquot removed for analysis with concentrated HCl ((2), foot-note 8) and by evaporation of acetic acid before rather than after hydrolysis, the decomposition of tyrosine can be minimized. Alternatively, the columns can be operated initially with volatile ammonium acetate buffers.

Results

The rate of hydrolysis of oxidized ribonuclease by chymotrypsin at pH 7.0 and 25°, measured by the ninhydrin method (5), is shown by the solid line in Fig. 1. The dash line gives for comparison the corresponding result obtained with trypsin under identical experimental conditions. Whereas the rate of hydrolysis of oxidized ribonuclease in the presence of trypsin becomes extremely slow after the first 6 hours, hydrolysis in the presence of chymotrypsin proceeds at an appreciable rate throughout the first 20 hours.

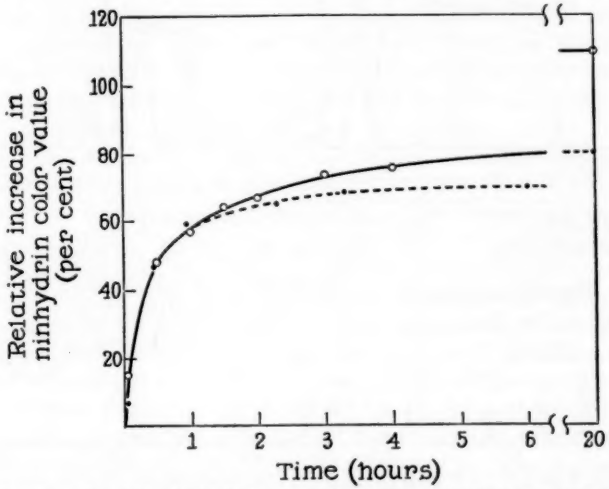


FIG. 1. The rate of the hydrolysis of oxidized ribonuclease (1 per cent solution) by chymotrypsin (○) and trypsin (●) at pH 7.0 and 25° (enzyme concentration, 0.005 per cent). The curves have been corrected for the small amount of autodigestion occurring in a control solution of the enzyme.

Since trypsin catalyzes the hydrolysis of twelve bonds (2), the result shown in Fig. 1 would indicate that the action of chymotrypsin has caused the hydrolysis of at least fifteen to sixteen peptide bonds, on the average, after 20 hours and that there is a considerable variation in the susceptibility of many of these bonds.

In view of the apparently less specific character of the action of chymotrypsin, the optimal conditions for hydrolysis were determined by analytical scale experiments in which columns of Dowex 50-X2 were employed to separate the peptides formed after progressively longer periods of exposure to the enzyme. As a result of these tests, a 24 hour hydrolysis was selected for the preparative scale experiments. After 24 hours, a number of pep-

tides shown to be present after shorter times of hydrolysis had completely disappeared, and the quantities of other peptides, present at first in small amounts, had increased to a maximum.

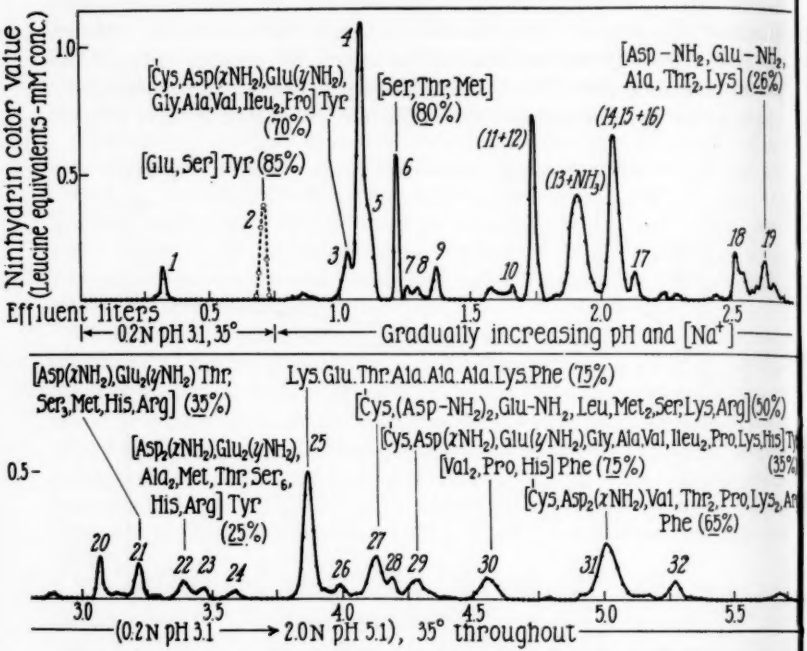


FIG. 2. The peptides in a 24 hour chymotryptic hydrolysate of oxidized ribonuclease. Chromatography of a hydrolysate from 200 mg. of protein was carried out on a 150 × 1.8 cm. column of Dowex 50-X2. The mixing chamber for the influent buffer had a volume of 2450 ml. The effluent was collected in 10 ml. fractions. Aliquots (0.5 ml.) were removed for analysis by the ninhydrin method. The dash line gives the ninhydrin color obtained after alkaline hydrolysis of aliquots of the effluent fractions. The figures in parentheses give the yield of each peptide. The sequence of the amino acid residues in brackets is undetermined.

The hydrolysis was repeated on a larger scale, with 200 mg. of oxidized ribonuclease, and the peptides obtained after 24 hours were separated as shown in Fig. 2. The results obtained after hydrolysis of aliquots of each of the effluent fractions with alkali have not been included on this curve, except in the region around 0.75 liter of effluent, where there was detected a peptide (Peak 2) which gave no color with ninhydrin before hydrolysis. No other major ninhydrin-negative peptides were found. The peak given by

ammonia (accumulated from the reagents during the preparation of the peptides) disappeared in the course of the alkaline hydrolysis to reveal the small peak arising from Peptide 13.

On the basis of the quantitative amino acid analyses given in Table I, the peaks marked 2, 3, 6, 19, 21, 22, 25, 27, 29, 30, and 31 in Fig. 2 appeared to arise from individual peptides. The amino acid composition of each of these peptides is given in terms of the integral number of residues of each constituent amino acid and is indicated in abbreviated form adjacent to the corresponding peak on the curve. In the formulation of those peptides containing phenylalanine or tyrosine residues, the assumption has been made, in accordance with the specificity studies of Bergmann and Fruton (*cf.* Green and Neurath (6)), that chymotryptic hydrolysis occurred at the carboxyl bond of the aromatic amino acid and that, therefore, these residues occupy carboxyl-terminal positions in the peptides.

The amino acid analyses indicated the presence of mixtures of peptides in the regions of the curve marked 4 + 5, 11 + 12, and 14 + 15 + 16. To separate these peptides, rechromatography under different experimental conditions of pH and temperature on columns 150 × 1.8 cm. was employed.

The partly separated mixture 4 + 5 in Fig. 2 was completely resolved into its components (Fig. 3, *A*) on a column operated at 50°, from which elution was effected with 0.2 N sodium citrate buffer at pH 3.1. The alkaline hydrolysis procedure was employed for the analysis of aliquots of the effluent fractions in order to conserve material. Rechromatography of the peptide mixture marked 11 + 12 in Fig. 2, on a column operated at 50° with a buffer of gradually increasing pH (3.1 to 5.1) as the eluent, gave the result shown in Fig. 3, *B*. Similarly, the mixture of peptides marked 14 + 15 + 16 in Fig. 2 was separated into its constituents, with the result shown in Fig. 3, *C*. The calculated yields for peptides that required rechromatography are minimal.

The analytical data for the peptides shown in Figs. 2 and 3 are summarized in Table I. For purposes of identification, the peptides listed in Table I are numbered as they appear in Figs. 2 and 3, with the added prefix O-Chy to denote that they have been derived by the chymotryptic hydrolysis of oxidized ribonuclease. These abbreviations will be followed hereafter. The compositions of the individual peptides in terms of the integral number of residues of the constituent amino acids per molecule are given in bold-faced type in Table I. All values for amino acids present at as much as 0.01 of a residue are included. It will be apparent that most of the peptide fractions secured from the chromatograms contain impurities, although the quantities, on a residue basis, rarely exceed 15 per cent, with the apparent exception of cysteic acid. As has been discussed previously (2), high values for cysteic acid are frequently obtained from pep-

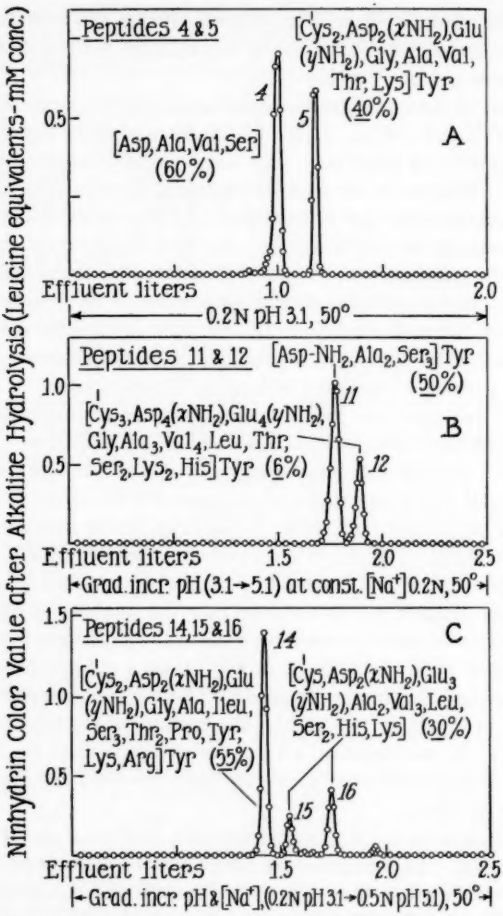


FIG. 3. Rechromatography of peptide fractions obtained from the chromatogram shown in Fig. 2. In each case chromatography was carried out on a 150 × 1.8 cm. column of Dowex 50-X2. A 2450 ml. mixing chamber for the influent buffer was used. The effluent was collected in 10 ml. fractions and 0.50 ml. aliquots were removed and subjected to alkaline hydrolysis prior to analysis by the ninhydrin method.

tides that have been hydrolyzed in the presence of excess buffer salts because of slight interference from material that emerges near the break out of the solvent front and reacts with ninhydrin to give a red color.

Of the peptides presented in Table I, only four require separate com-

The composition of each peptide fraction obtained from chromatograms of the type shown in Figs. 2 and 3 is expressed in terms of the molar ratios of the constituent amino acids as determined by chromatography on columns of Dowex 50-X4. Values for principal components given in bold-faced type. Values for amino acids present to less than 0.01 of a single residue omitted. Peptides numbered as in Figs.

Amino Acid Composition of Peptide Fractions Obtained from Tryptic Hydrolysate of Oxidized Ribonuclease

The composition of each peptide fraction obtained from chromatograms of the type shown in Figs. 2 and 3 is expressed in terms of the molar ratios of the constituent amino acids as determined by chromatography on columns of Dowex 50-X4. Values for principal components given in bold-faced type. Values for amino acids present to less than 0.01 of a single residue omitted. Peptides numbered as in Figs. 2 and 3. Peptides O-Chy 3 and 29 had to be hydrolyzed for 50 hours at 110° to liberate the full 2 residues of isoleucine per molecule.

Amino acid	Peptide O-Chy																	
	2	3	4	5	6	11	12	14	15	16	19	21	22	25	27	29	30	31
Aspartic acid	0.07	**1.09**	**0.99**	**2.05**	0.09	**1.08**	**3.96**	**2.11**	**2.11**	**2.10**	**1.08**	**1.13**	**2.05**	0.20	**1.98**	**1.03**	0.08	**1.97**
Glutamic "	**1.01**	**0.99**	0.02	**1.00**	0.05	0.11	**3.62**	**1.03**	**2.97**	**3.02**	**1.07**	**1.91**	**2.04**	**1.07**	**1.10**	**0.93**	0.09	0.06
Glycine	0.05	**0.95**		**1.02**	0.04	0.07	**1.29**	**0.97**			0.10		0.16		0.09	**0.93**	0.07	0.04
Alanine	0.05	**1.01**	**1.03**	**0.98**	0.08	**2.02**	**3.00**	**1.05**	**2.00**	**2.01**	**0.98**	0.23	**1.99**	**2.91**	0.16	**1.04**	0.05	0.06
Valine		**0.98**	**0.99**	**0.97**	0.04	0.09	**3.57**		**2.72**	**2.87**	0.04		0.10		0.07	**1.00**	**1.97**	**1.00**
Leucine	0.02		0.04	0.05	0.03	0.03	**0.98**	0.04	**1.09**	**1.03**	0.07	0.06	0.11	0.13	**0.82**	0.12	0.02	0.01
Isoleucine	0.01	**2.09**		0.05				**0.98**	0.07		0.06	0.06	0.05	0.02	0.03	**2.04**		0.01
Serine	**1.02**	0.11	**1.05**	0.06	**1.00**	**3.01**	**2.19**	**3.10**	**2.19**	**2.05**	0.12	**2.95**	**5.58**	0.24	**1.06**	0.17	0.08	0.03
Threonine		0.09	0.12	**0.95**	**1.05**		**1.04**	**1.85**			**1.89**	**1.26**	**1.06**	**1.00**	0.15	0.14	0.05	**1.92**
Cysteic acid	0.10	**1.04**		**1.96**	0.06	0.15	**3.21**	**2.09**	**1.33**	**1.31**	0.23	0.32	0.36	0.31	**1.16**	**1.15**	0.20	**1.13**
Met. sulfone					**0.95**						0.08	**0.89**	**1.11**	0.05	**1.81**	0.04		
Proline		**1.04**						**0.90**								**0.88**	**1.04**	**1.02**
Phenylalanine	0.02			0.04		0.02		0.02	0.10	0.08	0.03	0.02	0.04	**1.09**	0.06	0.08	**1.00**	**0.96**
Tyrosine	**0.97**	**0.90**		**0.98**		**0.90**	**1.03**	**2.00**			0.02	0.12	**0.71***			**0.78***		
Histidine			0.16			0.10	**1.08**		**0.90**	**1.03**	0.11	**0.86**	**0.92**	0.16		**0.91**	**0.97**	0.04
Lysine	0.01	0.07		**1.02**	0.02	0.05	**1.85**	**0.95**	**1.00**	**0.97**	**0.98**	0.22	0.19	**1.93**	**1.08**	**1.00**	0.06	**1.99**
Arginine								**1.03**			0.11	**1.00**	**0.96**	0.13	**0.99**			**1.03**
Amide-NH_3 (approx)	0.1	**0.8**	−0.1	**2.0**	0.0	**1.3**	**7.2**	**1.3**	**2.7**	**2.9**	**1.9**	**0.9**	**2.3**	0.3	**3.1**	**1.0**	0.1	**0.9**
Total No. of residues	3	10	4	11	3	7	27	18	16	16	6	10	17	8	10	12	5	11

* When tyrosine-containing peptides are eluted by concentrated sodium acetate-citrate buffers (>1 M), it is necessary to desalt the samples prior to hydrolysis in order to avoid decomposition of major quantities of tyrosine (see the text). The tyrosine determinations for O-Chy 22 and 29 still gave low values in spite of the fact that they were carried out on peptides that had been desalted.

ment. O-Chy 14 consists of 18 residues and contains 2 residues of tyrosine per molecule. Thus, 1 of the 6 tyrosine residues in ribonuclease (7) must be linked by its carboxyl group to an amino acid that confers chymotrypsin resistance to the bond. Since Bell (8) has shown that a -Tyr.Pro- bond in β-corticotropin is resistant to chymotrypsin, and since peptide O-Chy 14 contains a proline residue, it is possible that this same bond may be present in the peptide and be responsible for the stability of one of the tyrosyl linkages.

O-Chy 2, previously referred to in connection with Fig. 2, is a ninhydrin-negative tripeptide containing glutamic acid, serine, and methionine sulfone. The failure to give color with ninhydrin suggests that the peptide contains an amino-terminal pyrrolidonecarboxylic acid group, formed by the cyclization of an amino-terminal glutamine residue. Since the peptide was recovered in good yield, such a cyclization could not have taken place during chromatography, but probably occurred during the enzymatic hydrolysis or in the course of the storage thereafter at pH 2 before the mixture was chromatographed. Sanger, Thompson, and Kitai (9) have recently described a number of peptides containing amino-terminal glutamine residues, which were obtained from insulin, and which readily underwent cyclization to pyrrolidonecarboxylic acid derivatives. Such a reaction may underlie the observed difference in chromatographic behavior (Fig. 2) of the two glutamic acid-containing peptides, O-Chy 15 and 16, which have the same amino acid composition (Table I). The amide-NH_3 approximations are not accurate enough to be definitive on this point.

A number of peptide fractions from the chromatogram shown in Fig. 2 were obtained in too small a yield to permit precise amino acid analysis or represented mixtures of peptides obtained in low yields. These fractions are not included in Table I. Peak 1 arose from a peptide obtained in a yield of about 20 per cent, with the approximate composition Cys_2,Glu,Asp_2,Met_2, but contaminated with appreciable quantities of other acidic peptides. Since material moving near the break out of the solvent front is unlikely to be well resolved, no attempts were made to separate a pure peptide from the mixture. Peak 9 corresponded to a mixture of at least two large peptides obtained in yields of less than 10 per cent, and it was not investigated further. The broad zone including Peak 10 appeared to be composed of small peptides obtained in low yield. The analyses revealed that Peak 13 arose from a mixture of at least two peptides, obtained in about 8 per cent yield, containing leucine and valine, and related in composition to O-Chy 16. Peak 17 represented material analyzed for a 10 per cent yield of a large segment related to peptide O-Chy 16 but contaminated by a smaller quantity of another peptide. Peak 20 was composed largely of peptide O-Tryp 10 (*cf.* Fig. 2 (2)), obtained in 15 per cent yield, but contaminated

with at least one other peptide obtained in 4 per cent yield per molecule of oxidized ribonuclease. It is not possible to state whether or not this small amount of O-Tryp 10 was formed as a result of the action of the trace of trypsin that contaminated the chymotrypsin. The effluent fractions comprising Peaks 7, 8, 18, 23, 24, 26, 28, and 32 gave relatively small increases in ninhydrin color following hydrolysis, and the mixtures were not studied in detail.

DISCUSSION

Chymotryptic Hydrolysis—From the present state of knowledge concerning the specificity of chymotrypsin (*cf.* (6)), hydrolysis appears to occur preferentially at the peptide bonds involving the carboxyl groups of residues of the type —NHCH(CH_2R)CO—, where R is an aromatic or large neutral substituent. On this basis, the action of the enzyme on oxidized ribonuclease would be expected to be most rapid at the peptide bonds involving the carboxyl groups of the 6 tyrosine and the 3 phenylalanine residues in the peptide chain. Slower cleavages would be expected at the carboxyl groups of the 2 leucine residues, and perhaps at the carboxyl groups of the 4 methionine sulfone residues. No information as to the action of chymotrypsin on peptides containing methionine sulfone residues is available, but it is known (*cf.* (6)) that synthetic substrates containing methionine are split. Hydrolysis at other loci in oxidized ribonuclease might be anticipated, however, since Sanger and Thompson (10) found that a cysteic acid-serine bond in the glycyl chain of insulin is cleaved during chymotryptic hydrolysis.

The view that chymotrypsin will hydrolyze preferentially peptide bonds involving the carboxyl groups of the aromatic amino acids is supported by the data in Table I. The 3 phenylalanine residues in the peptide chain have been recovered in the three peptides O-Chy 25, 30, and 31, each obtained in yields of between 65 and 75 per cent per molecule of oxidized ribonuclease. The 6 tyrosine residues are present in five analytically distinct and unrelated peptides, O-Chy 2, 3, 5, 11, and 14, each obtained in yields of between 40 and 80 per cent per molecule of oxidized ribonuclease. O-Chy 22 and 29, obtained in lower yields, are less completely hydrolyzed tyrosine-containing peptides that will be shown to include the residues contained in O-Chy 11 and 3, respectively.

The fact that hydrolysis by chymotrypsin is not limited to bonds involving the aromatic residues, but also occurs more slowly at other points in the peptide chain of oxidized ribonuclease, is brought out by the data in Table I and in Figs. 2 and 3. O-Chy 6 contains only serine, threonine, and methionine sulfone, and in its formation a splitting at the carboxyl group of methionine sulfone probably occurred. Indication of relatively non-spe-

cific hydrolysis is also afforded by peptide O-Chy 19 (26 per cent yield), which contains no phenylalanine, tyrosine, leucine, or methionine sulfone, and which is subsequently shown not to arise from the carboxyl-terminal end of the chain. The sum of the number of amino acid residues recovered from the individual peptides listed in Table I is 151, excluding O-Chy 12, obtained in only 6 per cent yield, and counting O-Chy 15 and 16 as one sequence. This value considerably exceeds 124, which is the total number of residues known to be present in the molecule. There must, therefore, be certain portions of the peptide chain that appear in more than one of the peptide fragments isolated in relatively low yields (Figs. 2 and 3). For example, the methionine sulfone residues in peptides O-Chy 6, 27, 21, and 22 add up to 5, whereas there are only 4 methionine residues in the protein (7). The two peptides obtained in yields of 80 and 50 per cent, O-Chy 6 and O-Chy 27, contain 1 and 2 methionine sulfone residues, respectively. The 4th methionine residue is probably shared by the two peptides isolated in the lowest yields, O-Chy 21 at 34 per cent and O-Chy 22 at 26 per cent. The amino acid analyses of these two fragments indicate that they do come from the same portion of the molecule, with O-Chy 21 corresponding to O-Chy 22 from which a segment of 7 residues (O-Chy 11) has been split by further hydrolysis. For reasons such as these, the results obtained after chymotryptic hydrolysis are more difficult to interpret than those secured after the action of trypsin. The thirteen principal peptides of the O-Tryp series account for the whole molecule in terms of 124 residues and thus provide the soundest information to use as a basis for the derivation of a partial structural formula for the oxidized protein.

Partial Structural Formula for Oxidized Ribonuclease—A consideration of the results presented in this and previous communications has made it possible to propose as a working hypothesis a formulation that shows the manner in which the peptides produced by the action of trypsin, chymotrypsin, and pepsin are linked to one another in the original molecule of oxidized ribonuclease. In the derivation of this scheme (Fig. 4) certain assumptions have been made. It is assumed in the first place that the amino acid residues in oxidized ribonuclease are linked in a single unbranched chain containing peptide bonds between only α-amino and α-carboxyl groups. The evidence for the presence of a single chain has been presented by Anfinsen *et al.* (11), and there is thus far no indication that branching occurs. It has been assumed further that, in peptides resulting from the specific enzymatic action of trypsin (2), lysine or arginine, if present, occupies the carboxyl-terminal position. Similarly, it is assumed that tyrosine or phenylalanine, if present in peptides formed by the action of chymotrypsin, occupies the carboxyl-terminal position. The lysine- and arginine-free segment of 20 residues obtained by tryptic action (O-Tryp 16) is reserved for

inclusion as the carboxyl-terminal section of the chain. Allocation of the amide groups to asparagine and glutamine residues is postponed until the end of the derivation.

In deriving the arrangement shown in Fig. 4, it is simplest from the point of view of exposition to begin at the amino-terminal end of the chain and proceed stepwise towards the carboxyl-terminal end, linking, in appropriate order, the thirteen peptides obtained by tryptic hydrolysis. Actually, however, the same formula is derived irrespective of where the reconstruction is begun, and alternative arrangements for the principal segments have invariably led to impasses which prevented the development of a complete formula compatible with all of the experimental data.

O-Tryp 10–O-Tryp 15—The heptapeptide sequence Lys.Glu.Thr.Ala.-Ala.Ala.Lys (O-Tryp 10) occurs at the amino-terminal end of the molecule ((2), Anfinsen *et al.* (11)). An octapeptide corresponding in amino acid composition to O-Tryp 10 plus 1 residue of phenylalanine (O-Chy 25) has been isolated from the chymotryptic hydrolysate. From among the segments liberated by trypsin, O-Tryp 10 is the only possible source of 2 lysine and 3 alanine residues in a heptapeptide sequence. Hence, from the composition of O-Chy 25, phenylalanine must occupy the eighth position in the chain. Confirmatory evidence is provided by the isolation of O-Pep 9 (the same octapeptide as O-Chy 25) and O-Pep 6 (Fig. 4) from the peptic hydrolysates (3).

The second O-Tryp segment in the sequence must, therefore, contain a phenylalanine residue in the amino-terminal position. The 3 phenylalanine residues in ribonuclease are present in O-Tryp 9, 15, and 16. O-Tryp 16 is the carboxyl-terminal section of the molecule. In O-Tryp 9 phenylalanine has been found not to be amino-terminal by the dinitrophenyl method of Sanger. By elimination, therefore, the tripeptide O-Tryp 15 is shown to follow O-Tryp 10, and the known end sequence is extended to 10 residues by the addition of -Phe.Glu.Arg-.

O-Tryp 15–O-Tryp 4—The peptide of the chymotrypsin series that provides the linkage to the third O-Tryp segment must contain the sequence Glu.Arg at the amino-terminal end. O-Chy 14, 21, 22, and 27 contain glutamic acid and arginine residues. Two of these possibilities can be eliminated because the choice of either can be shown to lead to requirements that cannot be met by any of the remaining peptides of the trypsin series. The reasons for excluding O-Chy 27 are as follows: If the lysine-containing decapeptide O-Chy 27 followed O-Chy 25, it would have to encompass -Glu.Arg- plus either one complete lysine-containing O-Tryp peptide of 8 or less residues (O-Tryp 5, 6, 8, 11, or 14), or 1 of the 2 lysine residues of the longer peptide, O-Tryp 9. The first possibility is eliminated by the absence of both alanine and threonine from O-Chy 27, one or the other of

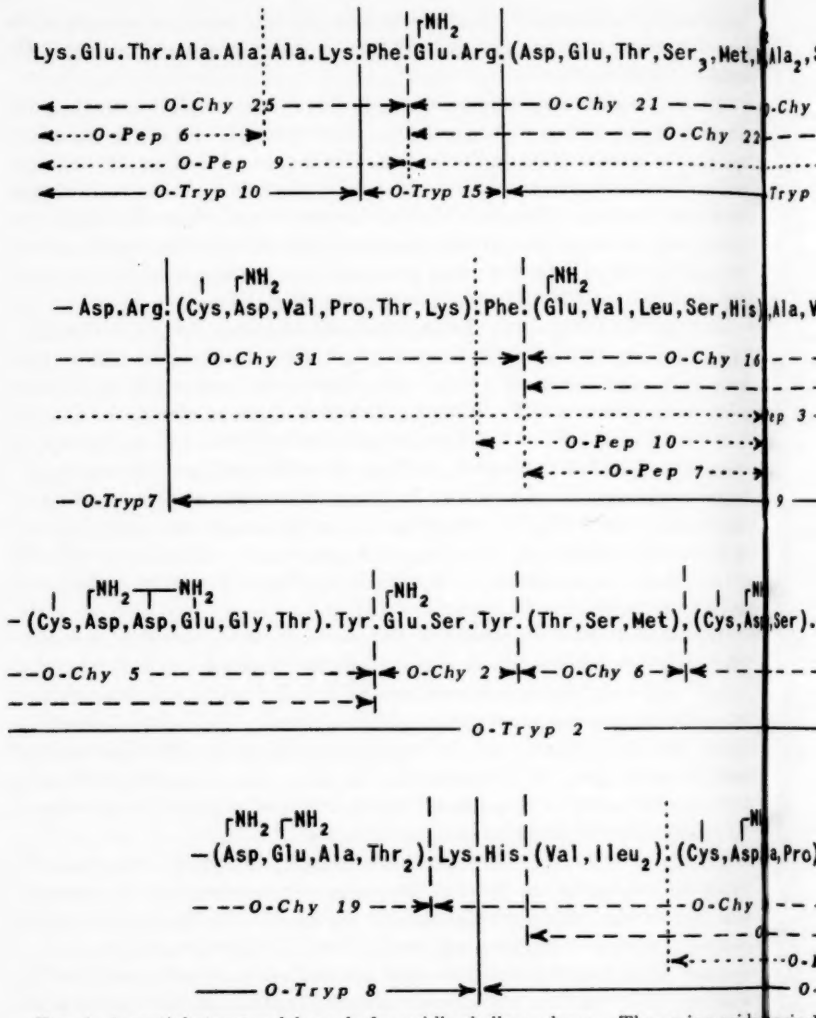
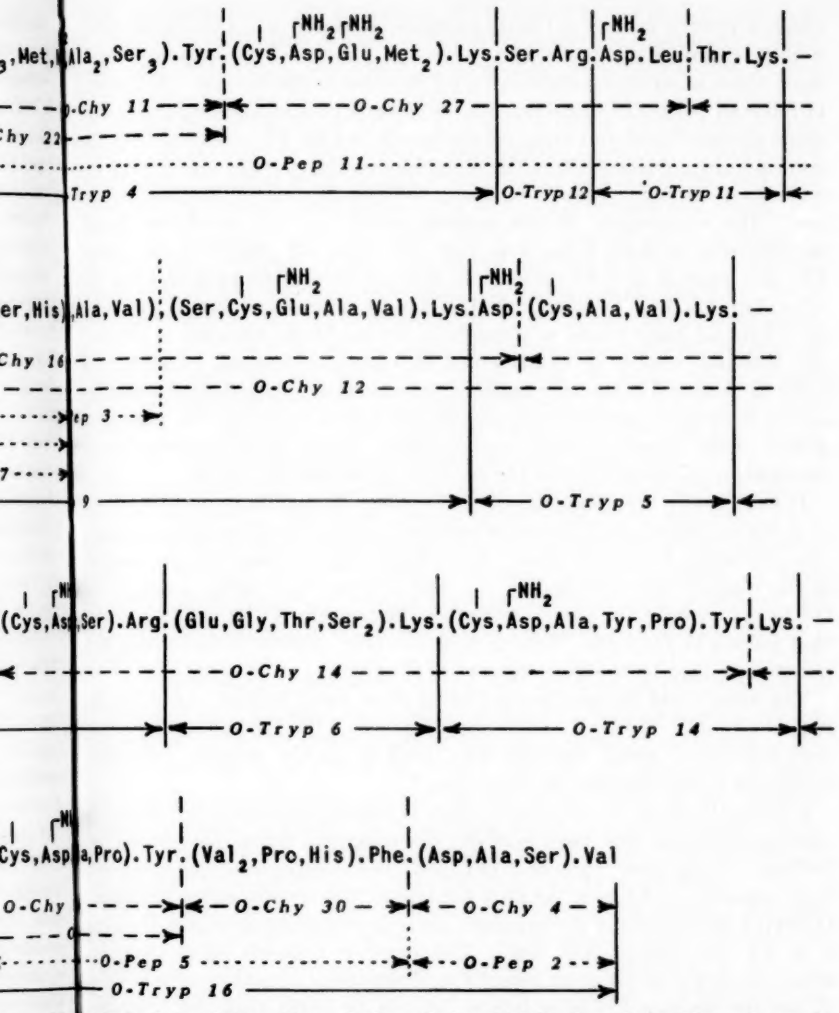


FIG. 4. A partial structural formula for oxidized ribonuclease. The amino acids in a singl of the studies of the peptides liberated by tryptic, chymotryptic, and peptic hydr quences mined. In the oxidized protein, each half cystine residue in the original protein is pr ic acid above an aspartic or glutamic acid residue indicates tentatively that the β- or γ-car present line, of chymotrypsin by the dash line, and of pepsin by the dotted line.



ino acids in a single chain of 124 residues, the formula for which has been derived from the results otic hydrolyses of the amino acid residues in parentheses, and set off by commas, are undeter- otein is present as the amide. The points of cleavage by trypsin are indicated by the solid

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which is present in each of the six peptides listed above. The second possibility requires 8 of the residues in O-Chy 27 and O-Tryp 9 to be coincident, which is impossible since O-Tryp 9 contains no methionine. If O-Chy 14 followed O-Chy 25, the -Glu.Arg- of O-Tryp 15 would correspond to the single glutamic acid and arginine residues in O-Chy 14. Since O-Chy 14 also contains a lysine residue, the remainder of this peptide would have to encompass an entire lysine-containing O-Tryp peptide of 16 residues or less. The composition of this peptide would have to be drawn from the following residues: Cys_2, Asp_2, Ileu, Thr_2, Ser_2, Gly, Ala, Tyr_2, Pro, Lys. The only peptide of the trypsin series that could be included is O-Tryp 14 (Cys, Asp, Ala, Pro, Tyr_2)Lys. If O-Tryp 14 were encompassed entirely by O-Chy 14, there would be required in O-Chy 14 the presence of 2 tyrosine residues, neither of which would be carboxyl-terminal. This possibility is rendered extremely unlikely, inasmuch as chymotrypsin usually causes hydrolysis preferentially at peptide bonds involving the carboxyl groups of the aromatic amino acid residues. From these considerations, therefore, it is concluded that O-Chy 21 or 22 follows O-Chy 25.

It has already been pointed out that O-Chy 21 and 22 apparently come from the same portion of the chain because they possess 10 residues in common, including 1 of methionine. Accordingly, the two peptides are placed in the manner indicated in Fig. 4. The presence of 6 serine residues in O-Chy 22 requires the same number in the peptide of the O-Tryp series that follows O-Tryp 15. O-Tryp 4, as the only peptide fulfilling this requirement, must be the next segment in the chain.

The amino acid composition of O-Chy 22 is equal to that of O-Chy 21 plus O-Chy 11. O-Chy 11 is the only peptide of the chymotrypsin series that contains 3 serine residues that could fit in the position following O-Chy 21 in the manner shown in Fig. 4.

O-Tryp 4–O-Tryp 12—O-Chy 27, since it contains lysine and 2 residues of methionine, is the only peptide of the chymotrypsin series that could link O-Tryp 4 to the next segment. O-Chy 27 is a decapeptide that also contains arginine, and hence the next peptide of the trypsin series following O-Tryp 4 must be one of the two short arginine-containing peptides, O-Tryp 7 or 12 (Ser.Arg or Asp.Arg). The decision between them depends upon whether a serine or an aspartic acid residue is available from O-Chy 27 for the position following lysine. It will be noted that the composition of O-Chy 27 also requires a leucine residue in the peptide of the trypsin series that comes after O-Tryp 7 or 12. Since there are only 2 leucine residues in ribonuclease, this requirement is important for the deduction of the sequence. The solution could have been reached by the performance of amino end-group analyses on the leucine-containing peptides, but an independent answer has come from the work of Anfinsen and Red-

field[3] who established the presence of the sequence Leu.Thr.Lys.Asp.Arg. This finding means that at least 1 leucine residue in the chain must precede Asp.Arg (O-Tryp 7), and hence it is Ser.Arg (O-Tryp 12) that follows O-Tryp 4.

O-Tryp 12–O-Tryp 11–O-Tryp 7–O-Tryp 9—Either of the two leucine-containing peptides, O-Tryp 9 or 11, could follow O-Tryp 12. The decision in favor of O-Tryp 11 is based jointly upon the findings of Anfinsen and Redfield referred to above and the isolation in 20 per cent yield of a peptide (O-Tryp 13 (2), Figs. 3 and 4), corresponding in composition to the sum of O-Tryp 9 and O-Tryp 7 (Asp.Arg), thus indicating that the two peptides occupy adjacent positions in the chain. If the leucine residue in O-Chy 27 were to come from O-Tryp 9, then O-Tryp 11 would have to be placed ahead of O-Tryp 7 in order to provide the leucine residue required by the peptide isolated by Anfinsen and Redfield. Under these circumstances, O-Tryp 9 and O-Tryp 7 could not be adjacent, as required by the composition of O-Tryp 13. This line of reasoning thus establishes the order O-Tryp 12, 11, 7, and 9 and carries the formulation through 61 residues.

The peptide of the chymotrypsin series that follows O-Chy 27 should contain residues of threonine, lysine, aspartic acid, and arginine. Only O-Chy 14 and 31 satisfy this requirement, and the former can be eliminated since it contains an isoleucine residue, whereas O-Tryp 9 does not. O-Chy 16 must follow O-Chy 31 because it is the only remaining peptide of the chymotrypsin series that can supply the leucine residue required by O-Tryp 9.

The composition of the segment from the 9th through the 51st residue corresponds to that found by Bailey *et al.* (3) for the large peptide (O-Pep 11) isolated from the peptic hydrolysate of oxidized ribonuclease. The unique composition of O-Pep 10, which contains phenylalanine, leucine, and histidine, and of O-Pep 7, which has the same residues except for phenylalanine, definitely places these peptides in the indicated position in O-Tryp 9. O-Pep 3 can be accommodated only in the latter half of O-Tryp 9, but its placement immediately adjacent to O-Pep 10 is arbitrary.

O-Tryp 9–O-Tryp 5—The composition of O-Chy 16 requires that the peptide of the trypsin series after O-Tryp 9 has aspartic acid in the amino-terminal position. There are four possibilities, O-Tryp 2, 5, 8, and 14. A clue is provided by the isolation of O-Chy 12 (Table I) which was ob-

[3] It is a pleasure to acknowledge the helpful interchange of information that has taken place between ourselves and Dr. Anfinsen and Dr. Redfield in the course of these studies. Their preliminary report on the sequence referred to above was presented before the meeting of the Federation of American Societies for Experimental Biology, San Francisco, April, 1955 (12). The full account of their independent studies on the structure of oxidized ribonuclease is given by Redfield and Anfinsen (13).

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tained in only 6 per cent yield, but which is analyzed for the sum of O-Chy 16 and O-Chy 5. If O-Chy 5 follows O-Chy 16 in the chain, the next peptide from the trypsin series must contain lysine and this eliminates O-Tryp 2. O-Chy 5 would have to contain 2 threonine residues to accommodate O-Tryp 8, or a proline and 2 tyrosine residues to accommodate O-Tryp 14. Since neither of these requirements is fulfilled, these two possibilities are eliminated and it is thus concluded that O-Tryp 5 follows O-Tryp 9.

O-Tryp 5–O-Tryp 2—After the placement of O-Tryp 5, the residual composition of O-Chy 5 requires the next peptide of the trypsin series to contain threonine and tyrosine, thus limiting the possibilities to only O-Tryp 2. The presence of isoleucine and arginine in O-Tryp 2 requires the next major peptide of the chymotrypsin series to be O-Chy 14, since 2 of the 3 isoleucine residues are assigned to the carboxyl-terminal segment (O-Tryp 16). The 4th and last methionine sulfone residue in O-Chy 6 definitely places this peptide as a subfragment of O-Tryp 2. O-Chy 2 is the only remaining serine- and tyrosine-containing peptide of the series and its assignment to the position indicated in Fig. 4 completes the accounting for O-Tryp 2. There is no evidence, however, as to whether O-Chy 2 precedes or follows O-Chy 6, and the order shown is provisional.

O-Tryp 2–O-Tryp 6–O-Tryp 14–O-Tryp 8—The lysine residue of O-Chy 14 must come from one of the three remaining lysine-containing peptides of the trypsin series, O-Tryp 6, 8, or 14. The 2 threonine residues in O-Tryp 8 eliminate it. O-Tryp 6 and O-Tryp 14 together account for all of the remainder of O-Chy 14 with 1 lysine residue left over. The indicated arrangement (Fig. 4), with O-Tryp 14 following O-Tryp 6, permits the assignment of 1 of the 2 tyrosine residues in O-Tryp 14 to a carboxyl-terminal position in O-Chy 14, and thus fulfils the specificity requirements of chymotrypsin. The placement of O-Chy 19 after O-Chy 14 permits the allocation of the sole remaining lysine-containing peptide of the trypsin series, O-Tryp 8. O-Chy 19 and O-Tryp 8 are isomeric peptides that possess identical amino acid compositions but are clearly different on the basis of their chromatographic behavior.

O-Tryp 8–O-Tryp 16—The only remaining lysine-containing peptide of the O-Chy series is O-Chy 29, and its placement after O-Chy 19 serves to connect the carboxyl-terminal segment, O-Tryp 16, with the rest of the chain. The presence of 2 isoleucine residues in both O-Chy 29 and O-Tryp 16 makes certain their close relationship to one another. O-Chy 3 also contains 2 isoleucine residues and, therefore, must overlap O-Chy 29.

The only two O-Chy peptides still unallocated, O-Chy 30 and O-Chy 4 account exactly for the 9 remaining amino acid residues in O-Tryp 16. O-Chy 4 has been assigned the carboxyl-terminal position on the basis of the data of Anfinsen *et al.* (11), who showed valine to be the carboxyl-ter-

minal residue in ribonuclease. The same peptide (Asp, Ala, Ser, Val) has been isolated by Anfinsen (14) and by Bailey *et al.* (O-Pep 2 (3)) from peptic hydrolysates. The fact that serine and valine are the last 2 residues in ribonuclease has been established by Niu and Fraenkel-Conrat (15) who employed hydrazinolysis. Redfield and Anfinsen (13) have determined that the full sequence is Asp.Ala.Ser.Val. The isolation of O-Pep 5 in 95 per cent yield by Bailey *et al.* (3) has provided evidence that requires 1 histidine, 1 valine, and both of the isoleucine residues to be the first 4 residues at the amino-terminal end of O-Tryp 16. O-Pep 5 is an isoleucine-free peptide of 12 residues which uniquely fits in the carboxyl-terminal segment, but contains only 1 of the 2 histidine and 2 of the 4 valine residues.

Allocation of Amide Groups—A tentative placement of amide groups has been undertaken by attributing to each peptide in Fig. 4 the number of amide groups required by the approximate values in Table I of Hirs *et al.* (2) for the peptides of the trypsin series, and in Table I of this communication for the peptides of the chymotrypsin series. An additional amide group is included in O-Chy 2, which is presumed to have contained originally an amino-terminal glutamine residue that underwent cyclization to pyrrolidonecarboxylic acid during the isolation. In spite of the very approximate nature of the amide-NH_3 estimations (*cf.* (2)), the data from the two series of peptides agree, with two exceptions. O-Tryp 9 accounts for four amide groups, whereas the O-Chy peptides from this same portion of the molecule account for only three of these groups. From the amide content of the peptides of the trypsin series that make up O-Chy 14, it would be expected to contain two amide groups, whereas analysis revealed only one and three-tenths. These discrepancies emphasize the provisional nature of the assignment of amide groups shown in Fig. 4. The allocation of only three groups to O-Tryp 9 reduces the total number of amide groups assigned to the trypsin series of peptides from eighteen ((2), Table I) to seventeen, a value which is in agreement with the number of amide groups estimated to be present by analysis of the intact protein (7).

Conclusions—The validity of some parts of the arrangement shown in Fig. 4 can be checked by submitting peptides of the trypsin series to the action of chymotrypsin and vice versa. Experiments of this type have been completed with two peptides. O-Tryp 4 has been isolated in a salt-free form and hydrolyzed with chymotrypsin, and the products have been separated chromatographically. Three peptides were obtained, one of which was indistinguishable from O-Chy 11. The other two had the expected compositions Asp, Glu, Thr, Ser_3, Met, His and Cys, Asp, Glu, Met_2, Lys. Similarly, three peptides were obtained upon chymotryptic hydrolysis of the carboxyl-terminal peptide (O-Tryp 16) which contains 20 resi-

dues. Two products were indistinguishable from O-Chy 4 and O-Chy 30, respectively, while the third contained the 11 residues (Glu, Asp, Gly, Ala, Val, $Ileu_2$, Pro, Cys, His, Tyr) which would be expected to arise from the amino-terminal end of O-Tryp 16.

It should be emphasized that the formula shown in Fig. 4 is a working hypothesis. More support is needed for the assignment of a position to some of the individual peptides, since a few of the fragments were obtained in only low yield. Nevertheless, the reconstruction shown appears to be unique in that it accommodates satisfactorily all of the data that have been accumulated thus far; none have been omitted. Final proof for the validity of the scheme requires the completion of work now in progress on the detailed sequence of the amino acid residues in the isolated peptides. Meanwhile, the partial structural formula shown in Fig. 4 permits the selection for detailed study of those peptides knowledge of which will reveal the most about the structure of ribonuclease. The scheme already shows certain features of the structure of the protein, and the results may thus facilitate study of the association of portions of the molecule with its enzymatic and physical properties.

SUMMARY

Oxidized ribonuclease has been subjected to the action of chymotrypsin and the mixture of peptides formed has been fractionated on columns of Dowex 50-X2 (150 × 1.8 cm.). Eighteen peptides containing from 3 to 27 amino acid residues have been obtained in yields ranging from 6 to 80 per cent, and each has been subjected to quantitative amino acid analysis on columns of Dowex 50-X4. These data, combined with similar results obtained previously after the action of trypsin and pepsin, have permitted the derivation of a partial structural formula for oxidized ribonuclease that shows, as a working hypothesis, how all the peptides formed by the action of trypsin, chymotrypsin, and pepsin can be arranged to form the original chain of 124 amino acid residues.

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ENZYMATIC FORMATION OF GLYCERYL AND PHOSPHOGLYCERYL METHYLTHIOL ESTERS

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Methyl mercaptan is a naturally occurring compound known to be formed and utilized by living organisms. It can arise in microorganisms (1) and in the rat (2) from methionine. It is rapidly oxidized in the rat, and its methyl carbon atom is incorporated into a number of compounds in this animal (3). In yeast cultures it promotes the accumulation of thiomethyl adenosine (4).

Reported here is a recently found enzymatic process involving MeSH.[1] Incubation of this substance with PGA in a crude yeast extract results in the formation of glyceryl methylthiol ester. The latter has been isolated from a reaction mixture and crystallized.

A study of the reactions involved in GMTE formation resulted in the separation of two enzyme fractions. One fraction catalyzes the formation of phosphoglyceryl methylthiol ester from PGA, MeSH, and ATP, as indicated in Reaction 1.

$$CH_3SH + {}^-OOC{-}CHOH{-}CH_2OPO_3^= + ATP \rightarrow CH_3SOC{-}CHOH{-}CH_2OPO_3^= + ADP + P \quad (1)$$

The second fraction contains a relatively specific phosphatase which hydrolyzes PGMTE to form GMTE according to Reaction 2 and does not act upon most of the other phosphate esters which have been tested.

$$CH_3SOC{-}CHOH{-}CH_2OPO_3^= \rightarrow CH_3SOC{-}CHOH{-}CH_2OH + P \quad (2)$$

In crude extracts thiol ester formation occurs with glycerate as the substrate as well as with PGA. Investigation revealed that a third separable enzyme is necessary in this case. This enzyme was found to catalyze PGA formation from glycerate according to Reaction 3. The reaction appears to be highly specific for glycerate.

$$ATP + \text{glycerate} \rightarrow PGA + ADP \quad (3)$$

[1] The abbreviations used are GMTE (glyceryl methylthiol ester), PGMTE (phosphoglyceryl methylthiol ester), PGA (3-phosphoglyceric acid), MeSH (methyl mercaptan), CoA (coenzyme A), P (inorganic orthophosphate), ATP (adenosinetriphosphate), ADP (adenosinediphosphate), and Tris (tris(hydroxymethyl)aminomethane).

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EXPERIMENTAL

Materials—Hydroxylamine with a low salt concentration was prepared according to Beinert *et al.* (5). The hydroxamic acids of glyceric, lactic, glycolic, and pyruvic acids were made by treatment of the corresponding ethyl esters with hydroxylamine by the method of Hestrin (6). Phosphoglycerohydroxamic acid was prepared enzymatically by incubating PGA, ATP, and hydroxylamine with phosphoglycerokinase.

PGA was obtained as the barium salt of the D isomer from the Nutritional Biochemicals Corporation, converted to the free acid with Dowex 50 (hydrogen form), and adjusted to pH 7.0 with KOH. Calcium DL-glycerate, obtained from the Bios Laboratories, Inc., was crystallized twice from water and converted to the potassium salt by the method used for PGA. MeSH, obtained commercially, was kept as a saturated aqueous solution (0.15 M) in contact with its crystalline hydrate at 0°. EtSH was similarly kept as a cold saturated aqueous solution.

CoA, Na ADP, and hexokinase were obtained from the Pabst Laboratories, crystalline Na_2 ATP, glucose-6-phosphate dehydrogenase, triphosphopyridine nucleotide, adenosine-5-phosphate, and *p*-nitrophenol phosphate from the Sigma Chemical Company, glucose-6-phosphate, fructose-1,6-diphosphate, protamine sulfate, and DL-homocysteine from the Nutritional Biochemicals Corporation, DL-glyceraldehyde and the dioxane complex of glyceraldehyde-3-phosphate from the Concord Laboratories, sodium glycerophosphate, containing about 25 per cent of the α isomer, and mercaptoethanol from the Eastman Kodak Company, and ribose-5-phosphate from the Schwarz Laboratories, Inc.

Phosphoglycerokinase was purified from yeast extract by a modification of the Bücher method (7). Pyruvate kinase and lactic dehydrogenase were generously supplied by Dr. J. Hurwitz.

Identification and Determination of Enzyme Reaction Products—The identity of PGMTE and GMTE rests largely on the isolation and characterization of the latter, described below, and the demonstrable enzymatic steps in its formation. In order to distinguish and identify these two compounds in small scale enzyme tests, they were converted to the corresponding hydroxamic acids, which were chromatographed on paper. 1 mmole of hydroxylamine was added per ml. to the enzyme reaction mixtures following incubation. After 20 minutes at room temperature 4 ml. of ethanol were added, the precipitate was removed by centrifugation, and the supernatant solution evaporated *in vacuo* to about 0.2 ml. 0.003 ml. of the derivative of GMTE was chromatographed on paper with water-saturated butanol according to Stadtman and Barker (8). The PGMTE derivative was diluted with water, passed through a column of Dowex 50 (hydrogen form), lyophilized, and chromatographed as the free acid with

water-saturated butanol. Both glycerohydroxamic acid and phosphoglycerohydroxamic acid spots were located with $FeCl_3$ (8). The former was also located by use of periodate (9), which detects glycols, and the latter by a phosphate reagent (10).

The R_F values for glycerohydroxamic acid and phosphoglycerohydroxamic acid were 0.16 and 0.05, respectively. The R_F values of compounds related to the glyceric acid derivative were 0.41 for lactohydroxamic acid, 0.23 for glycolohydroxamic acid, and 0.65 for pyruvohydroxamic acid. The values for the corresponding fatty acid derivatives are all 0.5 or larger (8).

GMTE and PGMTE were determined quantitatively by the hydroxamic acid method (11). The molar extinction coefficient of phosphoglycerohydroxamic acid was found as described below for the determination of PGA, and the corresponding value for glycerohydroxamic acid was determined to be 990 by using crystalline GMTE as a standard. ATP was determined by the method of Kornberg (12), ADP by the method of Kornberg and Pricer (13), and P by the method of Fiske and Subbarow (14).

PGA was identified and determined by incubation in the highly specific, purified PGA kinase system (7) with 0.5 M hydroxylamine, and the resulting phosphoglycerohydroxamic acid was determined by the method of Lipmann and Tuttle (11). The molar extinction of this compound in the Lipmann-Tuttle reagent at 540 mμ was estimated to be 775 on the assumption that 1 equivalent of P was released for each equivalent of hydroxamic acid. With as little as 0.5 μmole of PGA per sample, over 90 per cent conversion to hydroxamic acid was achieved in a 2 hour incubation in the presence of a relatively large amount of the enzyme. Identification of PGA was confirmed by a chromatographic method (10).

Preparation of Phosphoglyceryl Methylthiol Ester Synthetase—Yeast extract was prepared as described previously (15), except that the extraction time was reduced from 2 days to 16 hours. 0.1 volume of 1 M KH_2PO_4 was added to the extract, bringing the pH to 6.5. It was then heated to 63° in a boiling water bath and held at this temperature for 10 minutes. After rapid cooling and removal of the inactive precipitate by centrifugation, the solution, now at 0°, was adjusted to pH 4.5 with 2 M acetic acid. Sufficient solid $(NH_4)_2SO_4$ was added to make its concentration 70 per cent of saturation, and, after stirring 30 minutes in the cold, the precipitated enzyme was centrifuged. The precipitate was dissolved in a minimal volume of cold water and dialyzed for 6 hours against cold 0.01 M $KHCO_3$-HCl buffer, pH about 6.5.

The yield of enzyme was 70 to 80 per cent, purified 15-fold. This preparation is free of the two other enzymes described here. Estimation of the enzyme activity is made from the rate of thiol ester synthesis under the conditions described below.

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Preparation of Phosphoglyceryl Methylthiol Ester Phosphatase—To obtain an active extract, frozen bakers' yeast (Anheuser-Busch), prepared as previously described (15), was warmed to 0–3° and stirred slowly, without any addition, for 16 hours at this temperature. It was then centrifuged at 15,000 × *g* in the cold for 20 minutes. The packed cells were discarded, and the cold extract was brought to pH 4.5 by careful addition of 2 M formic acid. The precipitated enzyme, after centrifuging, was suspended by stirring in cold 0.1 M sodium acetate buffer, pH 4.5, double the volume of the original extract. After centrifuging again, the precipitate was suspended in cold water to 0.7 of the extract volume, and the pH was adjusted to 6.5 to 7.0 with 2 M Tris base. 0.2 volume of 2 per cent protamine sulfate was then added, and the suspension was centrifuged at 12,000 × *g*. The supernatant solution, still slightly turbid, was mixed with an equal volume of saturated $(NH_4)_2SO_4$ (0°), and the saturation of the resulting solution was brought to 65 per cent by further addition of solid $(NH_4)_2SO_4$. After stirring for 30 minutes, the precipitate was removed by centrifugation and the enzyme was precipitated by addition of sufficient $(NH_4)_2SO_4$ to bring the solution to 90 per cent of saturation. After 30 minutes the enzyme was centrifuged and dissolved in cold water to about 0.1 of the extract volume.

About 30 per cent of the enzyme activity was recovered, purified 10-fold. Its activity was determined from the increased P liberated in the presence of PGMTE synthetase. The preparation is quite free of non-specific phosphatase activity. It has been stored at −15° for several months with little loss of activity.

Preparation of Glycerate Kinase—Yeast extract was prepared as described above for PGMTE synthetase. Addition of 0.1 volume of 2 M formic acid to the cold extract, with stirring, brought the pH to about 4.0. The inactive precipitate was removed by centrifugation. The enzyme was then precipitated at 0° by slow addition, with vigorous stirring, of 0.06 volume of 6 N H_2SO_4, which lowered the pH to 2.6. The precipitate, after centrifugation, was suspended in cold water to a volume equal to one-third of the original extract, and the pH was adjusted to about 7.0 with 2.0 M Tris base. The suspension was stirred vigorously for 15 minutes and then centrifuged to remove inactive, insoluble material. The preparation may be dialyzed against cold, neutral buffer solutions and has been stored for several months at −15°. The yield of 6-fold purified enzyme is about 50 per cent.

This enzyme may be assayed by measuring ADP formation according to Reaction 3. A convenient assay in crude extracts, which contain a high PGA kinase activity, is to measure phosphoglycerohydroxamic acid formation by the combined action of the two enzymes on ATP, glycerate, and hydroxylamine.

Preparation and Properties of Glyceryl Methylthiol Ester—An extract of

bakers' yeast was prepared as described above for use in purifying PGMTE phosphatase. 0.25 volume of 2 per cent protamine sulfate was added to the cold extract with stirring, and the resulting precipitate was removed by centrifugation at 12,000 × *g* at 0°. The opalescent supernatant fluid was then dialyzed 4 to 5 hours against 200 volumes of 0.01 M $KHCO_3$-HCl buffer, pH 6.5 to 7.0.

A 500 ml. reaction mixture for the preparation of GMTE contained 100 ml. of the dialyzed preparation, 25 mmoles of PGA, 6 mmoles of MeSH, and 5.0 mmoles of $MgCl_2$. The pH was 7.0. ATP was generated by the action of glycolytic enzymes on a portion of the PGA and a trace of adenine nucleotide in the extract. The mixture, in a tightly stoppered 1 liter Erlenmeyer flask, was incubated for 2 hours at 30°. 100 ml. of 10 per cent metaphosphoric acid were then added, and, after removal of protein by centrifugation, the solution was brought to pH 7.0 by careful addition of 5 N NaOH.

The neutral solution was lyophilized and the dried residue dissolved and made up to a volume of 50 ml. with water. A small amount of insoluble material was removed by centrifugation. The preparation was then extracted continuously with ether for 12 hours with about 500 ml. of ether in the boiling flask. The clear ether solution was poured into a clean container to separate it from some insoluble substances, and the solvent was then removed by gentle boiling. When the residue was dried *in vacuo* over P_2O_5, GMTE crystallized as long needles on the walls of the flask. The crystals were dissolved in a small volume of dry ether and recrystallized by addition of petroleum ether. Some impurities usually contaminate the first precipitate of GMTE. If the supernatant solution is placed at −15°, large crystals slowly form. They may be dried *in vacuo* at room temperature. The yield was about 250 mg., m.p. 51°.

$[\alpha]_D^{20} + 85.3°$ (4 per cent in H_2O)

$C_4H_8O_3S$.[2] Calculated, C 35.3, H 5.9, S 23.5; found, C 35.6, H 6.3, S 23.6

In common with other thiol esters (16–18), GMTE has an intense ultraviolet light absorption maximum at 236 mμ (Fig. 1) which is destroyed by Na_2CO_3, $HgCl_2$, or NH_2OH. Loss of absorption with Na_2CO_3 or NH_2OH is associated with the appearance of free thiol, detectable with nitroprusside (19), and destruction by $HgCl_2$ is associated with precipitation of a mercaptide. The derivative formed with NH_2OH was identified chromatographically as glycerohydroxamic acid.

Results

Enzymatic Formation of Phosphoglyceryl Methylthiol Ester—Evidence for PGMTE synthesis according to Reaction 1 is presented in Table I. On

[2] Microanalyses were performed under the direction of Dr. W. C. Alford.

incubation of the complete enzyme system, P and PGMTE form in equivalent amounts, and the appearance of both is dependent upon MeSH, ATP, PGA, Mg^{++}, and enzyme. The rate of formation of P and PGMTE is related to the enzyme concentration (Table II) and to incubation time (Table III). Side reactions prevent the stoichiometric accumulation of ADP, which was found in amounts ranging from 60 to 80 per cent of the P and PGMTE values.

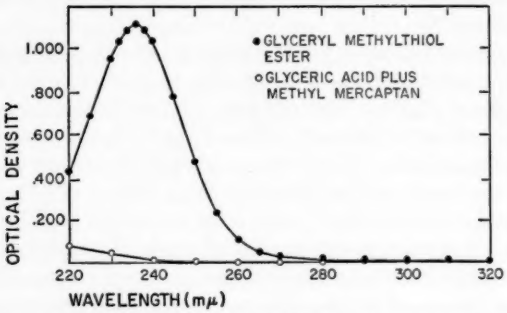

FIG. 1. Ultraviolet light absorption by GMTE. Measurements were made with a Beckman model DU spectrophotometer by using a quartz cell with a 1.0 cm. light path. The concentration of all of the indicated substances was 2×10^{-4} M in water. Readings were made against a water blank.

TABLE I

Enzymatic Formation of Phosphoglyceryl Methylthiol Ester and Associated Liberation of P

The complete system contained, in 1.0 ml., 100 μmoles of imidazole chloride buffer, 10 μmoles of $MgCl_2$, 12 μmoles of Na ATP, 8 μmoles of PGA, 12 μmoles of MeSH, and 2.8 mg. of enzyme. The pH was 7.0, the incubation time 30 minutes, and the temperature 30°.

	P	PGMTE
	μmoles per ml.	*μmoles per ml.*
Complete system	1.6	1.4
Omit MeSH	0.3	0.1
" ATP	0.0	0.0
" PGA	0.0	0.0
" $MgCl_2$	0.1	0.1
Boiled enzyme	0.1	0.1

With ethyl mercaptan or mercaptoethanol the rate of Reaction 1 is reduced to 40 and 20 per cent, respectively, of the rate with MeSH. Cysteine, homocysteine, glutathione, H_2S, and coenzyme A are inactive as substitutes for MeSH.

The reverse reaction has not yet been studied. The forward reaction is strongly inhibited by 0.1 M phosphate, arsenate, or hydroxylamine.

Enzymatic Dephosphorylation of Phosphoglyceryl Methylthiol Ester—On addition of PGMTE phosphatase to the PGMTE synthetase system, the phosphate ester in the PGMTE which is formed is immediately hydrolyzed. The P-thiol ester ratio in the reaction mixture after incubation is thus

TABLE II

Relation of PGMTE Synthesis and P Liberation to Enzyme Concentration

The conditions of the test were identical with those in Table I, except for the amount of enzyme used and the PGA concentration, which was 40 μmoles per ml. Each value represents the difference between the complete system and a control without MeSH.

Enzyme solution	P	PGMTE
ml.	*μmoles per ml.*	*μmoles per ml.*
0.03	1.5	1.3
0.06	2.6	2.4
0.10	3.6	3.4

TABLE III

Formation of Thiol Esters and P by PGMTE Synthetase Alone and with PGMTE Phosphatase

The conditions of this test were identical with those of Table I, except for the incubation time, the addition of enzymes as shown, and the PGA concentration, which was 40 μmoles per ml. 0.9 mg. of the phosphatase and 2.8 mg. of the synthetase were added per ml. Each value represents the difference between the complete system and a control without MeSH. All the values are micromoles per ml.

Incubation time	Synthetase		Synthetase plus phosphatase		Phosphatase	
	P	PGMTE	P	GMTE	P	Thiol ester
min.						
10	0.9	0.9	1.7	0.8		
20	1.6	1.7	3.6	1.7		
30	2.2	2.2	4.9	2.4	0.0	0.0

changed from 1 to 2, as shown by Reaction 4, which is the sum of Reactions 1 and 2. In the second and third columns of Table III are 1:1 results

$$PGA + MeSH + ATP \rightarrow GMTE + ADP + 2\ P \qquad (4)$$

with synthetase alone, and in the fourth and fifth columns, with both enzymes a P-thiol ester ratio of about 2:1. PGMTE phosphatase without the synthetase produces no significant amount of P or thiol ester (sixth and seventh columns, Table III). The thiol ester product of Reaction 1 has been identified as PGMTE and of Reaction 4 as GMTE.

The results in Table IV suggest that this phosphatase is quite specific for PGMTE. Of the phosphate esters tested, only phosphoglyceraldehyde and glycerophosphate were hydrolyzed to an appreciable extent by the enzyme preparation under the conditions of the test. The rates at which these two compounds were attacked were greatly reduced when their concentration was lowered to 1 μmole per ml. from the 5 μmoles per ml. concentration used in the test reported in Table IV. PGMTE, however, was generated in the reaction mixture, and its concentration was probably never

TABLE IV

Specificity of PGMTE Phosphatase

Each tube contained, per ml., 100 μmoles of imidazole chloride buffer, 10 μmoles of $MgCl_2$, 5 μmoles of the indicated substrate (other than PGMTE), and 12 μmoles of MeSH to simulate the conditions of the PGMTE test. The pH was 7.0 and the incubation time 30 minutes. The PGMTE value was taken as one-half of the net P liberated in a test identical with that of the fourth and fifth columns of Table III.

Substrate	P liberated
	μmoles per ml.
PGMTE	3.2
Glyceraldehyde-3-phosphate	1.9*
Glycerol phosphate	1.9
Ribose-5-phosphate	0.2
Fructose-1,6-diphosphate	0.0
Glucose-6-phosphate	0.0
Gluconate-6-phosphate	0.0
Glyceric acid-3-phosphate (PGA)	0.0
p-Nitrophenol phosphate	0.0
Adenosine-5-phosphate	0.0

* A non-enzymatic control was run with this compound.

TABLE V

Enzymatic Phosphorylation of Glycerate by ATP

The complete system contained, in 1.0 ml., 100 μmoles of imidazole chloride buffer, 10 μmoles of $MgCl_2$, 26.4 μmoles of Na ATP, 20 μmoles of K DL-glycerate, and 1.6 mg. of enzyme. The pH was 7.0, the temperature 30°, and the incubation time 30 minutes.

Experiment	ATP	ADP	PGA
	μmoles per ml.	*μmoles per ml.*	*μmoles per ml.*
Complete system	−5.4	5.6	5.4
Omit ATP			0.0
" glycerate	−0.6	0.4	0.0
" $MgCl_2$	−0.8	0.8	0.4
Boiled enzyme		0.4	0.0

as great as 0.1 μmole per ml. The affinity of the enzyme for PGMTE must therefore be relatively high.

Enzymatic Phosphorylation of Glycerate—The phosphorylation of glycerate, according to Reaction 3, is confirmed by the results in Table V. In the complete system ATP utilization is approximately equivalent to the amounts of ADP and PGA formed. Appearance of the products is dependent upon the presence of ATP, glycerate, Mg^{++}, and enzyme. ATP disappearance is similarly dependent upon other constituents of the system. This enzyme appears to be specific for glycerate, as no increase in ADP formation over the blank value could be detected when glycerol, gluconate, glycolate, DL-lactate, DL-glyceraldehyde, or GMTE was substituted for glycerate in the reaction.

DISCUSSION

The relevance of the reactions reported here to known biochemical processes is obscure. Pertinent information includes the fact that methyl mercaptan has been shown to be formed from methionine in several species (1, 2) and is thus known to be available for biochemical reactions. Schlenk and Tillotson have found that methyl mercaptan causes an accumulation of thiomethyl adenosine in yeast cultures (4). It seems possible that further study of the methylthiol esters reported here may link them with thiomethyl adenosine and methionine.

The relative specificity and high affinity of the phosphatase described here for PGMTE suggest that the hydrolytic reaction is not adventitious but may be a prerequisite for the metabolic use of the thiol ester.

A possible purpose for the phosphorylation of glycerate may be related to the utilization of GMTE. If the latter compound should prove to be a donor of the CH_3S— group, the glycerate portion of this molecule would become available for reconversion to PGA. A known enzymatic formation of D-glycerate occurs in the hydroxypyruvate-reducing system reported by Stafford *et al.* (20).

Reaction 1 appears, at least superficially, to be analogous to the enzymatic syntheses of thiol esters of CoA (21–23). No attempts to elucidate its mechanism have yet been made.

SUMMARY

1. An enzyme has been prepared from yeast which catalyzes the formation of phosphoglyceryl methylthiol ester, ADP, and P from PGA, MeSH, and ATP. Other known naturally occurring thiols such as cysteine, glutathione, and CoA are inactive as substitutes for MeSH in the reaction. EtSH and mercaptoethanol react in the system at 40 per cent and 20 per cent, respectively, of the rate found with MeSH.

2. A phosphatase has been prepared from yeast which appears to be rela-

tively specific for phosphoglyceryl methylthiol ester. It forms P and glyceryl methylthiol ester from this substrate.

3. Glyceryl methylthiol ester has been prepared enzymatically in 200 to 300 mg. quantities and crystallized, and its properties described.

4. A glycerate kinase has been isolated from yeast which catalyzes the formation of PGA and ADP from glycerate and ATP. This enzyme is highly specific for glycerate and causes no detectable reaction with other hydroxy acids or with glycerol.

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TERPENOID INTERMEDIATES IN THE BIOSYNTHESIS OF CHOLESTEROL*

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Previous reports from this laboratory (1, 2) have described a particle-free extract of rat liver which is capable of incorporating C^{14}-labeled acetate and β-hydroxy-β-methylglutaric acid (HMG)[1] into cholesterol. With this enzyme system, it has been possible to demonstrate that acetate and HMG can be incorporated into squalene, and that biosynthetic C^{14}-labeled squalene is incorporated into cholesterol. Supernatant fluid obtained following homogenization in phosphate buffer has been found to be capable of incorporating labeled HMG into squalene and farnesenic acid.[2]

Methods

Preparation of Farnesenic Acid

Farnesenic acid was prepared by the oxidation of farnesol (4) or nerolidol (5). Oxidation of either of these alcohols with dilute chromic acid yields farnesal. The aldehyde can be purified through the formation of the bisulfite addition product or directly oxidized to the acid. Since no better yield was obtained when the bisulfite compound was prepared, this step was omitted. The crude aldehyde was oxidized with alkaline silver oxide (6), and the resulting acid was extracted with ether after acidification. The ether solution was extracted with 1 per cent bicarbonate solution which was discarded. After extraction with 2 per cent KOH, the aqueous phase was acidified and the acid was reextracted with ether. After removal of the ether, the resulting material was chromatographed by reverse phase chromatography (described below). Elution of the acid was followed by titration of aliquots of each fraction. The first acidic peak, consisting of a mixture of more polar acids, was discarded. The fractions of the second, larger peak were combined and the solvent was distilled off under vacuum.

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[1] Abbreviations used in this paper are HMG = β-hydroxy-β-methylglutaric acid, AMP = adenosine monophosphate, DPN = diphosphopyridine nucleotide.

[2] Some of the evidence has been presented in a preliminary communication (3).

The resulting colorless oil gave a single peak on repeated chromatography and readily formed a crystalline salt with benzylpseudothiuronium chloride (7). The salt was crystallized from methanol-water and melted sharply at 122.5–123°. Total nitrogen analysis, found 7.04 per cent, calculated 7.08 per cent.[3]

Methyl-labeled β-hydroxy-β-methylglutaric acid was prepared from methyl C^{14}-acetate by the method described previously (2).[4]

Preparation of Enzyme Systems

The particle-free enzyme system was prepared as described previously (1).

The supernatant fluid was obtained after homogenization in phosphate buffer followed by centrifugation in a Spinco ultracentrifuge at 100,000 × *g* for 30 minutes. The clear supernatant solution was immediately lyophilized and the resulting powder was stored in a desiccator at −20°. Powders prepared and stored in this fashion were stable for a period of 6 weeks. Just prior to incubation, the powder was dissolved in ion-free water.

All incubations were carried on for 3 hours at 34° in an atmosphere of 95 per cent O_2-5 per cent CO_2. The substrates used were 1-C^{14}-acetate and methyl-C^{14}-HMG.

Recovery of Squalene and Other Carrier Substances

Squalene and other lipide-soluble materials were added to the incubation mixtures as emulsions in 2 per cent gelatin solution. These were prepared by addition of the gelatin solution to the compound followed by treatment in a high speed blender for 5 minutes. The emulsions were added to the incubation mixture after thermal equilibration of the flasks had occurred.

At the end of the incubation period, additional carrier compound or compounds and 10 volumes of 10 per cent KOH in 95 per cent ethanol were added to the flasks. The ethanol was evaporated on a boiling water bath under a nitrogen stream (duration of heating was approximately 30 minutes). The residual solution was acidified with dilute hydrochloric acid and extracted several times with ether. When compared with a 3 hour saponification, there was no significant difference in the recovery of carrier compounds or the specific activity of recovered labeled compounds.

The combined ether solutions were extracted twice with 2 per cent KOH

[3] The authors express their appreciation to Dr. Harry Vars for the Kjeldahl determinations.

[4] The authors express their appreciation to Dr. Joseph Rabinowitz, Radioisotope Division, Veterans Administration Hospital, Philadelphia, Pennsylvania, for the preparation of the labeled HMG.

and the ether was evaporated to dryness. The residue was dissolved in petroleum ether and chromatographed on alumina grade II.[5] From the petroleum ether eluates, squalene was obtained (9). Both farnesol and cholesterol could be obtained by eluting with 20 per cent ether in petroleum ether. Since one or the other, but not both, was sought in a given experiment, there was no interest in trying to separate a mixture. Cholesterol was further purified by preparation of the digitonide (10). Farnesol was chromatographed in the reverse phase system to be described below.

For recovery of farnesenic acid, the KOH extracts of the ether solution were acidified and extracted again with ether. After shaking with HMG solution and water, the ether was evaporated and the residue was chromatographed on the reverse phase column.

Chromatography of Farnesol and Farnesenic Acid

The reverse phase system described by Howard and Martin (11) for the separation of long chain fatty acids was found to be quite useful for the purification of these compounds. 6.7 gm. of Hyflo Super-Cel, prepared as described (11), were triturated with 6 ml. of hexane saturated with 80 per cent methanol. The mixture is put on a column 12 mm. in diameter and tightly packed under air pressure. The compound is introduced on the column and eluted with 80 per cent methanol saturated with hexane at the rate of 1 ml. every 2 or 3 minutes. After the column volume passed through, fractions were collected in tared planchets and counted. A typical curve for weight and activity is illustrated in Fig. 1.

On such a column, these compounds would consistently appear within one or two fractions on repeated chromatography.

Determination of Radioactivity

Compounds to be counted were dissolved in a suitable solvent and evaporated in stainless steel planchets under an infra-red lamp. The planchets were then dried in a vacuum desiccator; approximately 3 to 5 mg. of residue were usually obtained. The plates were counted in a gas flow windowless counter to 5 per cent error. All activities were corrected to infinite thinness.

Results

Following the incubation of the particle-free system with labeled acetate, radioactivity can be found in recovered cholesterol. If carrier squalene is added at the end of the incubation and subsequently recovered, there is variable activity in the squalene. However, the addition of the squalene

[5] Alumina, Woelm, neutral, grade I was converted to grade II by the addition of water (8).

emulsion at the beginning of the incubation results in a marked depression of the activity in cholesterol, and the recovered squalene has higher activity (Table I). Radioactive squalene obtained from several incubations and subsequently purified on alumina as described above was pooled and the mixed hexahydrochlorides were prepared (9). There was no change in the specific activity of the squalene on recrystallization of the derivative.

Biosynthetic squalene of high specific activity, which was prepared from labeled acetate as described above, was chromatographed on alumina and

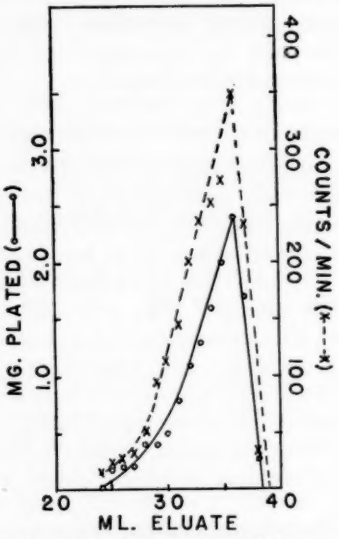

FIG. 1. Partition of radioactive farnesenic acid between 80 per cent methanol and hexane. The sample shown here was obtained from decomposition of benzylpseudothiuronium salt (see Table V). For the details of chromatography see the text.

subsequently incubated with the particle-free enzyme system (Table II). Carrier cholesterol, recovered as the digitonide, was found to contain significant activity.

It has been reported previously from this laboratory that the addition of citrate increases the incorporation of labeled acetate into fatty acids (12, 13). The effect of citrate upon the incorporation of labeled acetate into squalene was similar but not as marked (Table I). When HMG was used as a substrate, the addition of citrate appeared to inhibit or not affect the incorporation into squalene. The experiment reported in Table III is one of several showing the same drop in incorporation.

Comparison of the supernatant fluid with the particle-free extract (Table III) indicated that HMG was more efficiently incorporated into squalene

TABLE I

Incorporation of C^{14}-Acetate into Squalene and Cholesterol by Particle-Free Extracts of Liver

Each flask contained 7 ml. of particle-free extract and 1 mg. each of AMP, DPN, and sodium 1-C^{14}-acetate (3×10^6 c.p.m. per mg. of C). Where stated, potassium citrate in a final concentration of 0.01 M and 7.5 mg. of squalene in 0.5 ml. of emulsion were added. Incubated for 3 hours at 34°. Atmosphere = 95 per cent O_2-5 per cent CO_2. After incubation, 1 mg. of cholesterol and squalene to make a total of 15 mg. were added to each flask.

Experiment No.	Additions		Radioactivity of recovered carriers			
			Squalene		Cholesterol	
	Citrate	Squalene	Total activity	Substrate incorporated	Total activity	Substrate incorporated
			c.p.m.	*μmole*	*c.p.m.*	*μmole*
1	−	−	29	0.0008	80	0.0022
	+	−	83	0.0023	132	0.0037
2	−	−	36	0.0011	320	0.0089
	+	−	86	0.0024	590	0.0164
3	+	−	300	0.0080	315	0.0100
	+	+	640	0.0180	29	0.0008
4	+	−	63	0.0018	635	0.0176
	+	+	405	0.0110	266	0.0074

TABLE II

Incorporation of Biosynthetic C^{14}-Squalene into Cholesterol by Particle-Free Extracts of Rat Liver

The flask contents were the same as those in Table I, with the exception that all flasks contained potassium citrate in a final concentration of 0.01 M and 2 mg. of C^{14}-squalene in 0.5 ml. of emulsion. Conditions of incubation as in Table I. After incubation 1 mg. of cholesterol was added to each flask.

Experiment No.	Radioactivity of recovered cholesterol	
	Total activity	Substrate incorporated
	c.p.m.	*μmole*
1. Squalene, 3500 c.p.m. per μmole.....	110	0.034
2. " 860 " " "	32	0.037

by the former system. However, incorporation of HMG into cholesterol by this system is variable and is usually too low to be significant. Only supernatant fluid prepared from a phosphate buffer homogenate was active.

When supernatant fluid is prepared from a sucrose homogenate (0.25 M), the addition of the particulate fraction is necessary for activity.

When lyophilized supernatant fluid with labeled HMG as a substrate

TABLE III

Incorporation of Methyl-C^{14}-HMG into Squalene by Enzyme Systems from Rat Liver

Each flask contained 3 ml. of enzyme system, 5 mg. of potassium HMG (450,000 c.p.m. per mg. of C), and 0.3 mg. each of AMP and DPN. Where indicated, potassium citrate in a final concentration of 0.01 M was added. Each flask received 2 mg. of squalene in 0.2 ml. of emulsion. Conditions of incubation as in Table I. At the end of the incubation, an additional 3 mg. of squalene were added to each flask.

Enzyme system	Citrate	Radioactivity of recovered squalene		
		Specific activity	Total activity	Substrate incorporated
		c.p.m. per mg.	*c.p.m.*	*μmole*
Particle-free extract	+	41	205	0.0063
Supernatant fluid	+	132	660	0.0206
Supernatant fluid	+	101	505	0.0160
" "	−	170	860	0.0269

TABLE IV

Effect of Terpenoid Compounds on Incorporation of Methyl-C^{14}-HMG into Squalene by Supernatant Fluid from Rat Liver

The flask contents were the same as those in Table III except that each flask received 1 mg. of squalene in 0.1 ml. of emulsion. Specific activity of HMG = 176,000 c.p.m. per mg. of C. The terpenoid compounds were added prior to incubation as indicated. The alcohols were added in 0.1 ml. of gelatin emulsion and the acids in 0.1 ml. of a solution of the potassium salt. Conditions of incubation as in Table I. At the end of the incubation, 4 mg. of squalene were added to each flask.

Additions (1 mg. each)	Radioactivity of recovered squalene		
	Specific activity	Total activity	Substrate incorporated
	c.p.m. per mg.	*c.p.m.*	*μmole*
Control	107	535	0.042
Farnesol	14	70	0.005
Geraniol	81	405	0.032
Linalool	94	470	0.038
Control	117	585	0.046
Geranic acid	83	415	0.033
Control	102	510	0.040
Farnesenic acid	6	30	0.002

was employed, the rôle of various terpenoid compounds was studied. Geraniol, linalool, and farnesol were added as gelatin emulsions as described above. Geranic acid and farnesenic acid were added as solutions of the potassium salts. The 10-carbon compounds (geraniol, linalool, geranic acid) were without significant effect on the incorporation of HMG into squalene (Table IV).

The addition, however, of farnesol or farnesenic acid to the incubation mixture markedly reduced the incorporation of substrate into squalene. The recovered farnesol was chromatographed on the reverse phase column and contained no significant activity.

The recovered farnesenic acid was found to be radioactive. The acid collected from several incubations was pooled and treated as shown in Table V. It was found that repeated chromatography and preparations

TABLE V

Purification of C^{14}-Farnesenic Acid Recovered from Incubation Mixtures

Procedure	Specific activity, c.p.m. per mg. acid
Chromatograph	120
Benzylpseudothiuronium salt, m.p. 114–116°	135*
Recrystallize, m.p. 122–123°	158*
Decompose salt, recover acid	152
Rechromatograph acid (see Fig. 1)	148

* Corrected for weight of free acid.

of the analytically pure derivative resulted in no loss of specific activity. The derivative was decomposed and the recovered acid was chromatographed with no diminution of specific activity. It can be seen from Fig. 1 that the total weight and total activity of the serial fractions corresponded within the limits of experimental error.

DISCUSSION

Although there is some question concerning the rôle of squalene as a direct intermediate in the biosynthesis of cholesterol (14), the evidence presented by Langdon and Bloch (9, 15), as well as the experiments reported here, leaves little doubt that squalene is formed by liver enzymes and, furthermore, that it is readily converted into cholesterol. Although an oxygen-containing squalene (or squalenes) may be on the direct pathway, there is sufficient ground for the belief that a compound with the squalene carbon skeleton is involved in the biosynthesis of cholesterol.

The conversion of radioactive squalene to "cholesterol" has now been

demonstrated in an aqueous enzyme system. It should be noted that the method employed for the isolation of cholesterol does not rule out the possibility that part of the activity might have been due to lanosterol (16) or related compounds.

The incorporation of HMG into farnesenic acid demonstrates for the first time the possible involvement of a biologically active 15-carbon intermediate which might arise from the condensation of 3 isoprenoid units to yield a farnesyl type of structure. Again, as in the case of squalene, it is uncertain whether farnesenic acid is on the direct pathway or is in equilibrium with some direct intermediate. While the radioactivity of farnesenic acid can best be demonstrated by the prior addition of carrier, addition of the substance at the end of the incubation yields farnesenic acid with low but significant activity.

The inability of geraniol and geranic acid to reduce the incorporation of substrate into squalene suggests that the first condensation product of the 5- or 6-carbon precursor is not related to the geranyl group of compounds (17).

The failure to recover radioactive farnesol suggests either that it is not formed metabolically or that there is inadequate mixing of the carrier with the labeled material. This problem needs further investigation.

SUMMARY

A particle-free enzyme system prepared from rat liver is capable of incorporating C^{14}-acetate into squalene. The same system can convert squalene into cholesterol or closely related sterols.

When labeled β-hydroxy-β-methylglutarate is employed as a substrate, the supernatant fluid alone is capable of incorporating the substrate into squalene. By employing this system, it has been demonstrated that the addition of non-labeled farnesol or farnesenic acid can reduce the incorporation of substrate into squalene. The recovered farnesenic acid was shown to contain significant radioactivity.

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LACTIC DEHYDROGENASE

II. VARIATION OF KINETIC AND EQUILIBRIUM CONSTANTS WITH TEMPERATURE*

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Some time ago a study of the lactic dehydrogenase system was undertaken in this laboratory with the end in view that complete information about the kinetics of this system would be of value in establishing a mechanism of reaction for the simple dehydrogenases. In a preliminary report (1), the kinetic behavior of this system was interpreted in terms of a model, proposed by Laidler and Socquet (2), in which substrate and coenzyme are bound independently on the enzyme surface. The present, more precise data indicate that the system is better described by a model in which interaction of substrates on the enzyme surface is postulated.

Recently, Alberty (3) has extended the relation between the kinetic constants for an enzyme-catalyzed reaction and the equilibrium constant for the reaction to a variety of previously unconsidered reaction mechanisms. The present results have been tested by the relations found by Alberty (3) and, although the present data do not allow a choice to be made among several possible mechanisms, it can be concluded that the mechanism proposed by Theorell and Chance (4) for the alcohol dehydrogenase system is not applicable to lactic dehydrogenase.

EXPERIMENTAL

Lactic Dehydrogenase—Most of the determinations were made with crystalline lactic dehydrogenase prepared by the method of Straub (5). The

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Abstracted from portions of theses submitted by Miss Maire T. Hakala and by Mr. Andrew J. Glaid to the Graduate School of Duke University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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enzyme, recrystallized five times, exhibited the two electrophoretic components observed by Meister (6) and by Neilands (7). The component of higher mobility was separated from the slower component by compensated electrophoresis in phosphate buffer, pH 6.60, ionic strength 0.1. After one more recrystallization, the preparation was dialyzed free of ammonium ion against 0.1 M phosphate buffer, pH 7.20, and was dried by lyophilization. Since the lyophilized preparation, when stored in the cold, appears to lose no activity over a period of several months, it is a convenient source of enzyme for an extended series of measurements. However, since about 20 per cent of the activity of the crystalline enzyme is lost during lyophilization, determinations of the maximal velocity of the enzymatic reaction were made with a preparation which was kept as a crystal suspension in 0.5 saturated ammonium sulfate.

Enzyme concentrations were determined from optical density measurements at 280 mμ. The extinction coefficient at 280 mμ was found to be 1.45 cm. per mg. of protein per ml., assuming the nitrogen content of the enzyme to be 17 per cent (8). Neilands (7) has found this value to be 1.49, assuming a nitrogen content of 16 per cent.

Reagents—

Sodium pyruvate, prepared by the method of Robertson (9), was used for many of the earlier determinations. Later measurements were made with potassium pyruvate, prepared according to the procedure of Korkes *et al.* (10). Parallel measurements of reaction rate, made with these two preparations, failed to reveal measurable differences between them. In the presence of excess reduced diphosphopyridine nucleotide (DPNH) both preparations were completely reduced in the presence of lactic dehydrogenase.

For the equilibrium measurements and for some of the kinetic determinations, reagent DL-lactic acid was diluted with water, depolymerized by heating to boiling temperature for a few minutes, and neutralized against a glass electrode to pH 7.00 ± 0.05 with standard sodium hydroxide. Half the titration value was used to establish the concentration of L-lactate in the racemate. Most of the kinetic measurements, however, were performed with sodium L-lactate prepared by passing an Eastman preparation of zinc L-lactate or a preparation of calcium L-lactate from the California Foundation for Biochemical Research through a column of the acid form of Dowex 50, according to the procedure of Brin (11), followed by neutralization of the effluent with standard sodium hydroxide. In agreement with the findings of Kubowitz and Ott (8) and of Neilands (7), identical kinetic and equilibrium results were obtained with the L component of DL-lactate and with the pure L isomer.

Diphosphopyridine nucleotide (DPN) was a Pabst product, estimated

by the manufacturer to be of purity greater than 99 per cent. DPN was determined from optical density measurements at 260 mμ by using 18.0×10^3 cm. per mole per liter as the extinction coefficient (12, 13).

DPNH was prepared by the method of Ohlmeyer (14). As a corollary to the tests for purity of the pyruvate preparations, the extinction coefficient of this material was found to be 6.22×10^3 cm. per mole per liter at 340 mμ as determined by the procedure of Horecker and Kornberg (15). No reason can be assigned to our failure to duplicate this result in the earlier study (1).

In the presence of a large excess of pyruvate, over a 4-fold range of DPNH concentration, 10.5 ± 0.8 per cent of the initial absorption of DPNH solutions at 340 mμ did not disappear when lactic dehydrogenase was added to the system. Initial DPNH concentrations, determined at 340 mμ, were corrected by this factor. Whatever the nature of the impurity causing this residual absorption, it appears not to interfere with the enzymatic reaction since identical reaction velocities were found in parallel experiments with dithionate-reduced DPN and with DPN reduced by the action of crystalline alcohol dehydrogenase in the presence of a large excess of alcohol.[1]

Fresh solutions of all the reagents were prepared daily, with the exception of stock solutions of sodium L-lactate which were kept in the cold for not more than 5 days.

Methods—Spectrophotometric measurements were made with a Beckman model DU spectrophotometer equipped with dual thermospacers and with a cell compartment capable of accommodating 10 cm. cuvettes. Three walls of the cell compartment were of similar construction to the thermospacers, so that five of the walls of the cell compartment could be maintained at constant temperature by water circulated through them from a bath regulated to ±0.05°.

Reaction velocity measurements were made by determining the rate of change of DPNH concentration from the rate of change of optical density at 340 mμ. While some preliminary measurements were carried out in 1 cm. cuvettes, all measurements used for the evaluation of kinetic or equilibrium constants were made with 10 cm. cuvettes.

Stock solutions of the reagents were kept in the regulated bath for the duration of kinetic experiments, with the exception of the enzyme solution which was kept at room temperature if the bath temperature was higher than room temperature. For velocity measurements from the pyruvate side of the reaction, appropriate volumes of buffer, enzyme solution, substrate, and water, such that the final volume would be 50 ml., were added to a 125 ml. Erlenmeyer flask in the regulated bath. At zero time the

[1] This determination was carried out by Mr. Yasuo Takenaka.

coenzyme solution was added rapidly with swirling, and the system was transferred to the test cuvette. The initial optical density measurement was made 15 to 20 seconds after the coenzyme had been added and, thereafter, at 15 second intervals for 3 minutes. Since enzyme concentrations were adjusted to levels such that optical density readings during at least the 1st minute of reaction varied linearly with time, initial reaction velocities, determined from the initial slopes of plots of optical density against time, were not subject to serious error. The control cuvette contained the components present in the test cuvette with the exception of coenzyme. The final buffer concentration was 0.05 M sodium phosphate, pH 6.80.

The same procedures were followed in making velocity measurements from the lactate side of the reaction with the exception that neutralized hydroxylamine was added to a final concentration of 0.01 M. Preliminary measurements, in a system containing sufficient lactate and DPN (of the order of 10^{-3} M with respect to each reactant) to give a reasonably linear reaction course for a few minutes in the absence of a trapping agent for the pyruvate formed, indicated the initial reaction velocity to be increased 4.5 per cent in the presence of 0.01 M hydroxylamine. Since this difference is of the order of the error of the determination of initial reaction velocity, hydroxylamine was judged to be a suitable trapping agent for pyruvate under the conditions of our experiments.[2] Measurements of pH were made with a Beckman model G pH meter.

Results

At the outset of these studies, a series of measurements was made to outline the general properties of the system.

Stability of Enzyme—Solutions of the lyophilized enzyme preparation containing 0.02 γ of enzyme nitrogen per ml. in 10^{-3} M histidine buffers were found not to lose any activity at 30° in 1 hour if the pH of the buffer was between 6 and 10. At pH 4.8 and 11.2 nearly all the activity was lost. If the buffered solutions of enzyme were incubated at 54.5° for 1 hour, the pH range of maximal stability was narrowed to pH 7 to 9, and even within this range 25 per cent of the initial activity of the enzyme was lost.

Effect of Ion Concentration and Species on Reaction Rate—When sodium chloride was added to the 0.05 M phosphate buffer in which determinations of reaction velocity were routinely performed, it was found that the rate

[2] The failure to observe the hydroxylamine inhibition, recently studied by Kaplan and coworkers (16, 17), with liver alcohol dehydrogenase may arise, in part, from a fortunate choice of the order of addition of reagents, since enzyme, hydroxylamine, and DPN were not incubated in the absence of substrate, and, in part, from a smaller susceptibility of lactic dehydrogenase to this form of inhibition.

of reduction of pyruvate was not significantly altered by concentrations of added sodium chloride up to 0.05 M. However, when the molarity of added sodium chloride was increased to 0.2, 0.3, and 0.5, the reaction rate increased to 148, 164, and 175 per cent, respectively, of the mean rate observed when the concentration of added sodium chloride was less than 0.05 M.

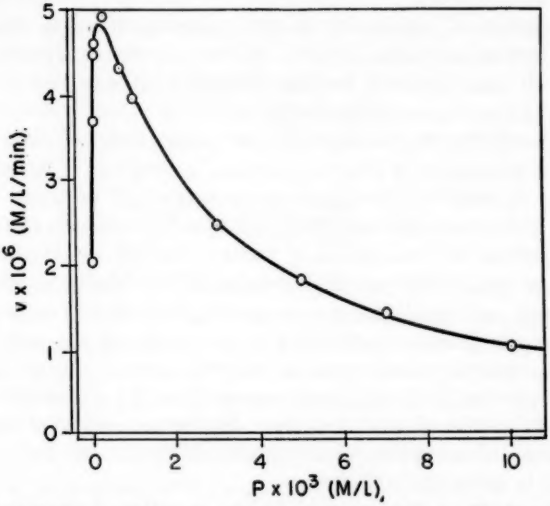

FIG. 1. Inhibition of the lactic dehydrogenase-catalyzed reduction of pyruvate by high concentrations of pyruvate. The curve was calculated from the equation of Lineweaver and Burk (19) for substrate inhibition with values of 1.8×10^{-5} for the Michaelis constant for pyruvate, 2.5×10^{-3} for the dissociation constant for the 2nd bound molecule of pyruvate, and 6×10^{-6} mole per liter per minute for the maximal velocity. The points are experimental points. The DPNH concentration was 1.29×10^{-5} M, the lactic dehydrogenase concentration approximately 0.02 γ per ml., and the temperature 27.5°. The determinations were made in 0.05 M phosphate, pH 6.80.

Replacement of the sodium phosphate buffer by potassium phosphate buffer caused no detectable change in the velocity of the reaction.[1] Furthermore, no change in reaction velocity was observed when the phosphate buffer was replaced by 0.9 per cent sodium chloride solution adjusted to pH 6.80.

Inhibition by High Concentrations of Reactants—In agreement with the observations of Kubowitz and Ott (8) and of Neilands (18), it was found that pyruvate, in high concentrations, is a powerful inhibitor for the reaction. Fig. 1 illustrates the behavior of a system containing 1.29×10^{-5} M DPNH in the presence of increasing concentrations of pyruvate.

No inhibition by DPNH was found when the DPNH concentration was increased to 4.56×10^{-4} M at a pyruvate concentration of 1.7×10^{-3} M, but when the pyruvate concentration was 2.5×10^{-4} M some decrease in reaction velocity was found at DPNH concentrations greater than 6×10^{-5} M. A detailed study of the reciprocal effects of pyruvate and DPNH with regard to substrate inhibition is being made as part of an investigation of inhibition in this system.

Concentrations of L-lactate up to 0.2 M were not found to cause inhibition at a DPN concentration of 1.3×10^{-4} M and, conversely, no inhibition was observed when the DPN concentration was raised to 1.65×10^{-3} M at a constant lactate concentration of 7.8×10^{-4} M.

Evaluation of Kinetic Constants—Prior to each series of determinations used for the estimation of kinetic constants, several sets of determinations were made to establish the approximate magnitude of these constants. The range of concentration over which suitable determinations can be made is limited because determinations of reaction velocity, made at concentrations of a reactant which are high relative to the Michaelis constant for that reactant, yield small changes in reaction velocity and consequent uncertainty in the graphical estimation of the Michaelis constant. On the other hand, determinations made at concentrations of a reactant which are low in relation to its Michaelis constant yield low absolute reaction velocities and increased relative errors. Further, as indicated above, high concentrations of pyruvate, relative to the Michaelis constant for pyruvate, result in substrate inhibition.

The present data appear to fit a kinetic equation of the form (3, 20)

$$\frac{V_{max}}{v} = 1 + \frac{K_A}{A} + \frac{K_B}{B} + \frac{K_{AB}}{AB} \qquad (1)$$

where v is the observed initial reaction velocity and V_{max} is the maximal reaction velocity when substrates A and B are present at high concentrations relative to their respective Michaelis constants, K_A and K_B. K_{AB} is a complex constant whose meaning in various possible reaction schemes is given by Alberty (3). In the case in which there is no compulsory binding sequence for the substrates and in which the equilibrium kinetic approach is used, K_{AB} is the dissociation constant for the ternary complex, EAB, into free enzyme, free A, and free B. Since it is clear from the work of several investigators (3, 20, 21) that the nature of the constants, K_A, K_B, and K_{AB}, depends upon the postulated reaction mechanism, and since the present results do not permit a choice to be made among several possible mechanisms, it seems pointless at this time to enlarge upon the possible meanings of these constants.

In a typical case, the kinetic constants were estimated from the present

data by making Lineweaver-Burk (19) plots of reciprocal initial reaction velocity against reciprocal pyruvate concentration for several constant levels of DPNH concentration. For each level of DPNH, the data describe a line having an intercept at $1/P = 0$ of $1/V_{max}(1 + K_R/R)$ and a slope of $1/V_{max}(K_P + K_{PR}/R)$.[3] If the intercepts are plotted against corresponding reciprocal values of DPNH concentration, the resulting linear plot has an intercept at $1/R = 0$ of $1/V_{max}$ and a slope of K_R/V_{max}. Determinations of initial reaction velocity in which the concentration of DPNH is varied at several constant levels of pyruvate concentration can be used in a similar way for the estimation of K_P and V_{max}. Typical plots of experimental data are shown in Figs. 2 and 3.

The complex constant K_{PR} was estimated by rearranging Equation 1 into the form

$$\frac{V_{max}}{v} - \left(1 + \frac{K_P}{P} + \frac{K_R}{R}\right) = \frac{K_{PR}}{PR} \qquad (2)$$

Since all the terms in the left-hand member of Equation 2 can be determined, a plot of the left-hand member of this equation against $1/PR$ should give a straight line of slope K_{PR}.[4] The value of K_{OL} is estimated in a similar way from data obtained from the lactate side of the reversible reaction. Since the values found for K_{PR} and K_{OL} depend upon the values assigned to the other kinetic constants, it is clear that errors of estimation tend to accumulate in the values of the complex constants. A typical plot used for the determination of K_{OL} is presented in Fig. 4.

In order to avoid the effects of substrate inhibition, it is clearly desirable to make estimates of Michaelis constants from the data obtained with substrate concentrations of the order of the Michaelis constants. On the other hand, the estimation of V_{max} from velocities measured at such low levels of substrate concentration involves a double extrapolation from points of relatively low precision. Self-consistent relative values for V_{max}

[3] Pyruvate, lactate, DPN, and DPNH have been abbreviated, in the kinetic treatment, as P, L, O, and R. K_P, K_L, K_O, and K_R are the corresponding Michaelis constants and K_{PR} and K_{OL} the complex constants for the reactions from the pyruvate and from the lactate side of the equilibrium, respectively.

[4] Although several investigators have concluded that a reversible, enzyme-catalyzed reaction involving two forms of substrate and two forms of coenzyme will give rise to curvilinear Lineweaver-Burk plots if the system behaves as a steady state system, and if there is no compulsory binding order for the reactants (3, 20, 22), calculated plots for a variety of assumed ratios of rate constants indicate that the curvilinearity will usually be too small to be experimentally detectable (23). It follows that the best test for the fit of experimental data to Equation 1 is the demonstration that a consistent value of K_{AB} is found over a wide range of variation of the product AB.

at various temperatures were obtained by making triplicate determinations of initial reaction velocity with each of three independently prepared

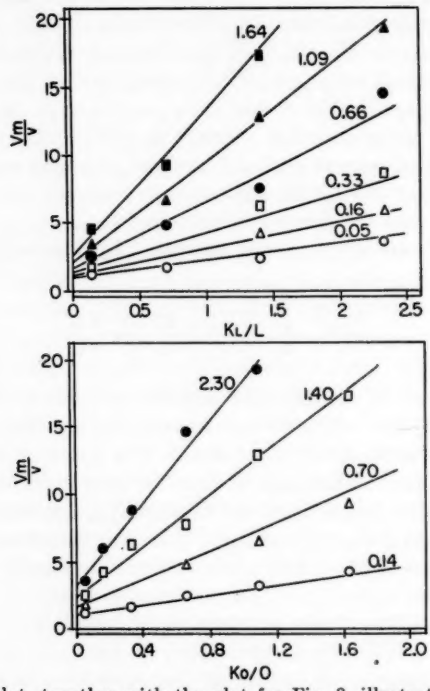

FIG. 2. This plot, together with the plot for Fig. 3, illustrates the variation of reciprocal initial reaction velocity, expressed as V_{max}/v, with reciprocal initial substrate or cosubstrate concentration, expressed as the ratio of the Michaelis constant for the reactant to the concentration of the reactant. The numbers above the lines in the figures indicate the ratio of the Michaelis constant to the concentration of the reactant whose concentration was held constant. The lines are calculated from Equation 1 by using the values given for the kinetic constant in Tables I and II. Since a change in the intercept in the upper graph of Fig. 2 and of Fig. 3 corresponds to a change in slope in the lower graph, the graphs must be taken together as a representation of the fit of the calculated lines to the experimental points. The reaction was measured from the lactate side at 29°. The value of V_{max} was 1.40×10^{-6} mole per liter per minute, and the lactic dehydrogenase concentration was approximately 1.6×10^{-2} γ per ml.

enzyme solutions at four temperatures with a single initial concentration of substrate and coenzyme. V_{max} for each temperature was then determined from Equation 1 by substitution into this equation of the values

for the initial substrate concentrations, for the mean initial reaction velocity, and for the Michaelis constants at each temperature. The values of V_{max} obtained in this way agreed well with the mean values found at various temperatures by the double extrapolation procedure.

The absolute value found for V_{max} at 25° was obtained from measurements made in a 1 cm. cuvette at 25° at a high concentration of DPNH

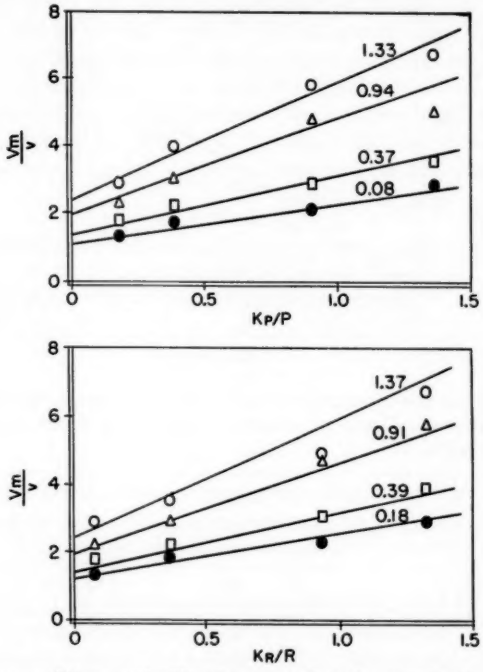

FIG. 3. Same conditions as in Fig. 2, except that the reaction is measured from the pyruvate side at 11.5°. The value for V_{max} was 7.14×10^{-7} mole per liter per minute, and the enzyme concentration was about 1×10^{-2} γ per ml.

(1.19×10^{-4} M) with pyruvate concentrations varying from 6 to 23 $\times$ 10^{-5} M. A preparation of crystalline, rather than lyophilized, enzyme was used for this determination, and the values found with lyophilized enzyme were adjusted to the value found with the crystalline preparation. The value of V_{max} found under these conditions, 4.15×10^4 moles per liter per minute per 135,000 gm. of enzyme (18), agrees well with turnover numbers which have been reported previously (6, 18).

The kinetic constants estimated from the data obtained at different tem-

peratures are summarized in Tables I and II. While it would have been desirable to make determinations from the pyruvate and lactate sides of the reaction at identical temperatures, the reaction velocity from the lactate side was too low at temperatures less than 15° to permit of reliable measurement.

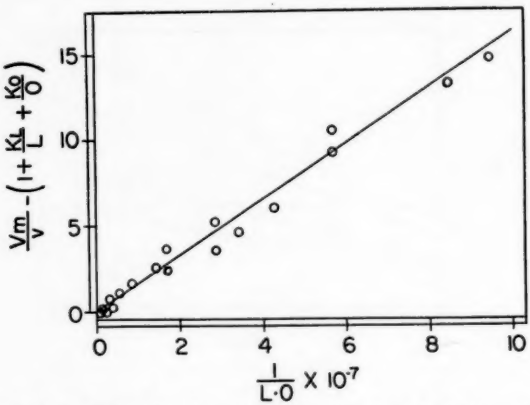

FIG. 4. A plot of the left-hand term of Equation 2 against the reciprocal of the product of substrate and coenzyme concentrations. This figure is drawn from the data presented in Fig. 2.

TABLE I

Kinetic Constants for Lactic Dehydrogenase-Catalyzed Conversion of Lactate to Pyruvate

Temperature	K_L	K_O	K_{OL}	V_{max}*
°C.	10^{-4} M	10^{-5} M	10^{-7} M	10^{4}
15	2.1	2.1	1.5	0.54[4]
22	5.3	2.5	1.7	0.95[1]
29	8.8[5]	2.7[5]	1.5	1.4[3]
36	20	3.0	2.4	2.2[4]

* V_{max} is expressed as moles per liter per minute per 135,000 gm. of enzyme.

Evaluation of Equilibrium Constant—The position of the equilibrium constant was found by determining the stable optical density reading after known initial concentrations of pyruvate, lactate, DPN, DPNH, and enzyme were placed in a 10 cm. cuvette in the temperature-regulated cell compartment. The reactions were carried out in 0.05 M phosphate, pH 6.80. Since the equilibrium constant for this reaction has been estimated previously (7, 8, 24), it was possible to calculate initial concentrations of

reactants which would give a suitable optical density reading at equilibrium. The enzyme concentration was of the order of 10^{-9} M. This enzyme concentration is somewhat higher than that used for reaction velocity determinations, but so much smaller than the equilibrium concentration of any reactant that preferential binding of one reactant would not be expected to influence the equilibrium constant in any measurable way. At each temperature the initial concentration of lactate was varied over a 16-fold range at two levels of initial DPN concentration. The initial concentrations of pyruvate and DPNH were equal in each case. In determining the equilibrium concentration of DPNH from the optical density measurement at 340 mμ, account was taken of the fact that at pH 6.80 the molar extinction coefficient of DPN is 43.0. Since the equilibrium concentration of DPN was high, compared to the equilibrium concentration of

TABLE II

Kinetic Constants for Lactic Dehydrogenase-Catalyzed Conversion of Pyruvate to Lactate

Temperature	K_P	K_R	K_{PR}	V_{max}*
°C.	10^{-6} M	10^{-7} M	10^{-13} M	10^4
11.5	2.7	7.1	3.7	1.1^9
16	5.5	16	5.0	1.8^4
25	15	35	6.5	4.1^5
35	34	62	34	9.6^5

* V_{max} is expressed as moles per liter per minute per 135,000 gm. of enzyme.

DPNH, the contribution of DPN to the equilibrium optical density was appreciable.

In agreement with the observation of Kubowitz and Ott (8), the apparent equilibrium constant for the reaction was found to be dependent on the initial concentration of lactate. This phenomenon is illustrated in the first four columns of Table III for the data obtained at 16°. Examination of the data led to the hypothesis that the lactate used in these measurements contained traces of some material reducible by DPNH in the presence of lactic dehydrogenase. This hypothesis was tested by measuring the decrease in absorption at 340 mμ when lactic dehydrogenase was added to systems which were 2×10^{-3} M with respect to L-lactate, and 4×10^{-6} M with respect to DPNH. Solutions of sodium lactate, freshly prepared from Merck DL-lactic acid, were found to contain 0.052 per cent of reducible material, while solutions freshly prepared from calcium L-lactate, supplied by the California Foundation for Biochemical Research, contained 0.007 per cent of reducible impurity. It was further observed that, when a stock solution of sodium L-lactate was allowed to stand in

air at room temperature for 2 weeks, the concentration of impurity rose from 0.007 to 0.2 per cent. The rate of oxidation of DPNH by the impurity was found to be diminished in the presence of hydroxylamine. Since lactic dehydrogenase is specific for α-keto acids, it seems probable that the impurity is pyruvate.

The data used for calculation of the equilibrium constants were corrected by adding 0.052 per cent of the initial L-lactate concentration to the initial pyruvate concentration. No account was taken of the trivial diminution

TABLE III

*Determination of Equilibrium Constant for Lactic Dehydrogenase-Catalyzed Conversion of Lactate to Pyruvate at 16°**

DPN_0	L_0	Change in DPNH concentration at equilibrium (Δ)	Apparent K_{eq} $\frac{(P_0+\Delta)(DPNH_0+\Delta)}{(L_0-\Delta)(DPN_0-\Delta)}$	$P_0 + 5.2 \times 10^{-4} / L_0\ (P_0')$	Equilibrium constant $\frac{(P_0'+\Delta)(DPNH_0+\Delta)}{(L_0-\Delta)(DPN_0-\Delta)}$
10^{-5} M	10^{-3} M	10^{-6} mole per l.	10^{-5}	10^{-3} M	10^{-5}
7.37	2.00	−5.17	0.436	7.04	0.981
7.37	4.00	−5.03	0.299	8.08	0.940
7.37	8.00	−4.83	0.218	10.16	0.994
7.37	16.00	−4.60	0.157	14.32	1.09
7.37	32.00	−4.59	0.079	22.64	1.01
10^{-4} M					
7.37	1.00	−3.31	0.978	6.52	1.16
7.37	2.00	−2.34	0.906	7.04	1.16
7.37	4.00	−1.46	0.697	8.08	1.02
7.37	8.00	−0.17	0.586	10.16	0.986
7.37	16.00	+1.00	0.415	14.32	0.907

* The initial concentration of DPNH ($DPNH_0$) and the nominal initial concentration of pyruvate (P_0) were each 6.00×10^{-6} M for each determination.

of initial L-lactate concentration by the trace impurity. The effect of this correction is shown in the last two columns of Table III.

In order to weigh the points obtained at moderate optical density readings more heavily than those obtained at very high or very low readings, the equilibrium constants were taken as the slopes of lines obtained by plotting the product of the equilibrium concentrations of pyruvate and DPNH against the product of the corresponding concentrations of lactate and DPN. These plots appear in Fig. 5.

DISCUSSION

The values for the various Michaelis constants in Tables I and II tend to be slightly smaller than those found by other investigators. In the region of neutrality and at temperatures between 20–27°, K_P has been reported as 9×10^{-5} (8), 5.2×10^{-5} (6), and of the order of 10^{-5} (7); K_i

has been estimated as 5×10^{-6} (8), less than 10^{-5} (6), and of the order of 10^{-5} (7); K_L and K_O have been estimated to be of the order of 10^{-3} and 10^{-5}, respectively (7). The explanation for the lower values found in this study may lie in the fact that, for a system obeying Equation 1, the estimation of K_A from a conventional Lineweaver-Burk plot of reciprocal

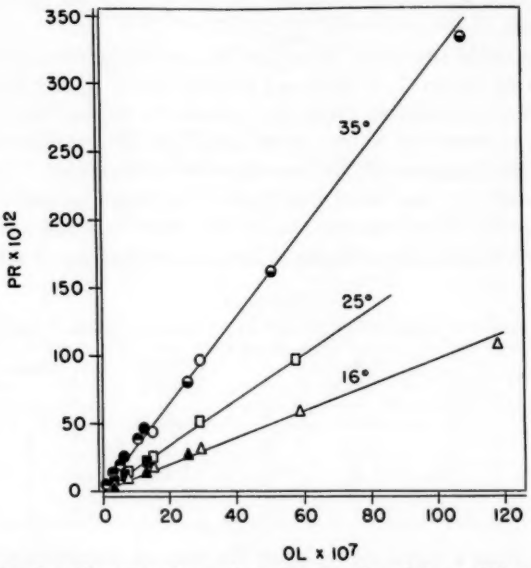

FIG. 5. The plots illustrate the method of evaluation of the equilibrium constants at various temperatures. Solid symbols, initial DPN concentration of approximately 7.4×10^{-5} M; open symbols, initial DPN concentration of approximately 7.4×10^{-4} M. Both refer to initial DPNH and pyruvate concentrations, uncorrected for the pyruvate present in the lactate, of 6.00×10^{-6} M. ◓, initial DPN concentration of 7.4×10^{-5} M; ◒, initial DPN concentration of 7.4×10^{-4} M for zero initial concentrations of DPNH and pyruvate. About one-third of the experimental points have been omitted to prevent crowding of the figure. The values of the equilibrium constants, determined as the slopes of these lines, are given in Table IV.

initial reaction velocity against reciprocal initial concentration of substrate A at a single constant concentration of substrate B yields, as the ratio of slope to intercept, not K_A, but $(K_AB + K_{AB})/(K_B + B)$. When B is large compared to K_B, the apparent value of K_A determined in this way approaches $K_A + K_{AB}/B$. Ingraham and Makower (22) have considered the variation of apparent Michaelis constants and maximal velocity with the concentration of the reactant, the concentration of which is held invariant in a determination.

The values in Tables III and IV for the equilibrium constant were cal-

culated from the expression, (pyruvate)(DPNH)/(lactate)(DPN). Since the complete reaction is lactate + DPN $\rightleftharpoons$ pyruvate + DPNH + H^+ (24), the complete expression for equilibrium constant is (pyruvate)(DPNH)(H^+)/(lactate)(DPN). If the term for hydrogen ion concentration is introduced into the value shown for the apparent equilibrium constant, the true equilibrium constant at 25° is found to be 2.76×10^{-12} mole per liter.

The relation of the value found for the equilibrium constant to previously reported values (7, 8, 24) is not entirely clear. It has been indicated that traces of a reducible impurity, present in the lactate preparations used, tend to cause low values to be found for the equilibrium constant when only the change in DPNH concentration is measured. On the other hand, Neilands (7) has found that lactic dehydrogenase parallels the behavior of alcohol dehydrogenase (25) in that the observed equilibrium constant is increased in the presence of high concentrations of enzyme. In-

TABLE IV

Fit of Kinetic and Equilibrium Constants to Relations Derived by Alberty (3)

Temperature	$\frac{V_f K_{PR}}{V_r K_{OL}}$	$\frac{V_f^2 K_P K_R}{V_r^2 K_L K_O}$	Observed equilibrium constant
°C.	10^{-5}	10^{-9}	10^{-5}
16	1.15	5.29	0.978
25	1.81	5.66	1.74
35	2.85	6.26	3.24

terpolation from a van't Hoff plot of the data of Kubowitz and Ott (8), obtained at 20° and 39°, yields a value of 4.16×10^{-12} for the equilibrium constant at 25°. The value reported by Racker (24), 4.4×10^{-12} at 25°, is in good agreement with that derived from the results of Kubowitz and Ott (8), but no enzyme concentration is specified for either of these determinations. Neilands (7) has recently found the mean value of the equilibrium constant at enzyme concentrations less than 10^{-6} M to be 3.3×10^{-12} between pH 7 and pH 10. There appears, however, to be a slight trend to these results with increasing pH, since the values found increase from 2.3×10^{-12} to 4.3×10^{-12} over this pH range.

The apparent equilibrium constant at 25°, corrected to pH 7.00, has a value of 2.76×10^{-5}. This value yields -0.135 volt for $\Delta E'_0$ for the system. If -0.32 volt is taken as the value of E'_0 for DPN-DPNH (26), E'_0 for pyruvate-lactate is -0.185 volt. Burton and Wilson (26) calculated the pyruvate-lactate value to be -0.190 volt from the equilibrium data of Racker (24).

The variation of the logarithm of the equilibrium constant with reciprocal absolute temperature, illustrated in Fig. 6, may be used to calculate

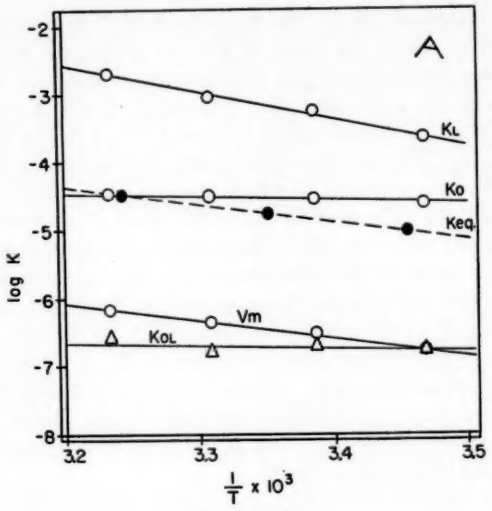

FIG. 6, A

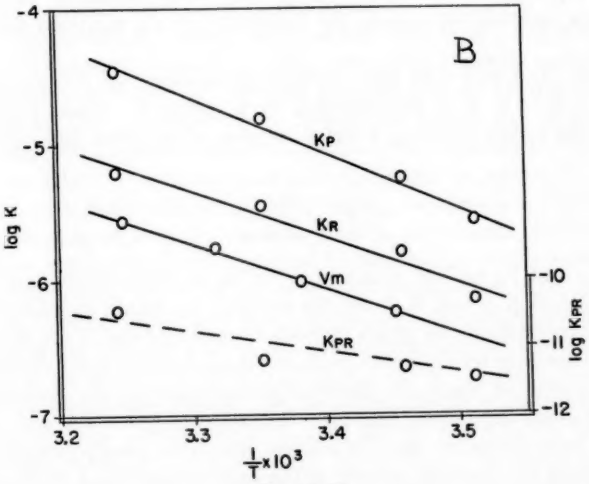

FIG. 6, B

FIG. 6. *A* and *B*. Variation of the logarithms of the kinetic constants and of the equilibrium constant with reciprocal absolute temperature. The lines were determined by the method of least squares. The values of K_P, K_R, and K_{PR} at 16° were given half the weight of the other points because of a somewhat poorer fit of the constants to the experimental points in plots of the type shown in Fig. 2.

an apparent value of ΔH for the reaction of 11.2 ± 0.2 kilocalories per mole. However, since the value of pK′ for phosphate buffer decreases with increasing temperature, the determinations were not made at constant hydrogen ion concentration. The value of pK′ for the buffer at various temperatures was obtained by interpolation in the data of Hitchcock and Taylor (27), and the values found for the equilibrium constants at 16° and 35° were corrected to a hydrogen ion concentration corresponding to a pH of 6.80. Use of these corrected values yields a value of 10.3 ± 0.2 kilocalory per mole for ΔH. This result is in remarkably good agreement with the value of 10.6 kilocalories per mole recently determined at pH 7.30 by direct calorimetric measurement by Katz (28), who estimates the uncertainty of his determination to be about 2.5 per cent.

From the true equilibrium constant found at 25°, $\Delta F°$ can be calculated to be 15.8 kilocalories per mole. From this result and that found for ΔH, ΔS for the reaction is −18.4 calories per mole degree. Katz (28) has discussed the thermodynamic properties of the reaction in terms of the formal partial reactions which are involved.

The relation between equilibrium and kinetic constants for enzyme-catalyzed reactions was originally derived by Haldane (29). Alberty (3) has recently extended Haldane's treatment to a number of previously unconsidered reaction mechanisms. The present data appear to be the first which are suitable for testing the usefulness of the relations derived by Alberty.

Alberty (3) found that four reaction mechanisms for the general reaction $A + B \rightleftharpoons C + D$ are described by Equation 1. These mechanisms all give rise to the following relation between equilibrium and kinetic constants:

$$K_{eq} = \frac{V_f K_{CD}}{V_r K_{AB}} \tag{3}$$

where V_f and V_r are the maximal velocities in the forward and reverse directions and K_{AB} and K_{CD} are the complex constants mentioned previously. The mechanisms which fit both Equations 1 and 3 are as follows: (*a*) the instance in which there is no compulsory binding order of substrates by the enzyme, in which the dissociation constants for the dissociation of A and B from the binary enzyme-substrate complexes *E*A and *E*B are different from those for the dissociation from the ternary complex *E*AB, and in which the limiting velocity is the rate of conversion of the ternary complex *E*AB to the ternary complex *E*CD; (*b*) the instance in which there is a compulsory binding order for the substrates, in which there is no single limiting reaction velocity, and in which the two ternary complexes, *E*AB and *E*CD, have sufficient stability to be of kinetic significance; (*c*)

a situation similar to that of (*b*), with the exception that only one ternary complex is postulated to be of kinetic significance; and (*d*) the mechanism, postulated by Theorell and Chance for the alcohol dehydrogenase reaction (4), in which one binary complex, *e.g.* *E*A, reacts so rapidly with substrate B that the possible ternary complex *E*AB cannot be detected by kinetic measurements. Alberty (3) also found that case (*d*), in addition to satisfying Equation 3, satisfies the relation

$$K_{eq} = \frac{V_f^3 K_C K_D}{V_r^3 K_A K_B} \tag{4}$$

The fit of the present data to Equations 3 and 4 is presented in Table IV. Since the various constants were not evaluated at identical temperatures, the values compared in Table IV were taken from the least squares lines, shown in Fig. 6, which relate the logarithms of the various constants to reciprocal absolute temperature. Considering the uncertainty of estimation of the kinetic constants, the agreement between the experimentally observed equilibrium constants and equilibrium constants calculated from the kinetic constants by the use of Equation 3 may be fortuitous. None the less, it is concluded from the present results that Equation 4 does not fit the data, and that the reaction mechanism which gives rise to Equation 4 is not a probable mechanism for this reaction.

The alternative mechanisms which satisfy Equations 1 and 3 all involve postulated ternary complexes. The postulation of such complexes has recently been criticized on the basis that there is no valid experimental evidence for their existence (30). While the present results provide no more than presumptive evidence in favor of this hypothesis, there appears to be a variety of corroborative evidence which is consistent with the existence of ternary complexes. The direct, stereospecific transfer of hydrogen between substrates and coenzymes in the alcohol and lactic dehydrogenase systems has been explained by Westheimer and Vennesland and their collaborators (31, 32) in terms of ternary complexes. An alternative, although improbable, explanation of these results is that hydrogen is transferred from the reduced form of one substrate to the enzyme and that this reduced enzyme reacts with the oxidized form of the other reactant. If binary complexes are assumed to be intermediates in each step of this reaction, the kinetic equation is $V_{max}/v = 1 + K_A/A + K_B/B$, and the equilibrium constant is given by $K_{eq} = V_f^2 K_C K_D / V_r^2 K_A K_B$ (3). Since the present results do not satisfy these relations, this possibility can be dismissed.

The change in the spectrum of DPNH in the presence of large quantities of lactic dehydrogenase, observed by Chance and Neilands (33), appears to provide direct evidence for a binary enzyme-DPNH complex. An en-

zyme-pyruvate complex seems to be a necessary postulate to explain the competitive behavior of oxamate with respect to pyruvate and lactate and the non- or uncompetitive behavior of this inhibitor with respect to DPN and DPNH (34).[5] In view of these indications that enzyme-substrate and enzyme-cosubstrate complexes exist, the postulate of a ternary enzyme-substrate-cosubstrate complex does not seem improbable.

At the present time, no choice can be made among the remaining possible reaction mechanisms. It is anticipated that studies now in progress may enable such a choice to be made. In view of the uncertainty attending the meaning of the individual kinetic constants, the interpretation of the variation of these constants with temperature (Fig. 6) must be deferred.

SUMMARY

The kinetic and equilibrium constants for the lactic dehydrogenase system have been evaluated over a range of temperature at pH 6.80. The initial reaction velocities, starting from either side of the reversible reaction, obey a kinetic equation of the form: $V_{max}/v = 1 + K_A/A + K_B/B + K_{AB}/AB$. The kinetic and equilibrium constants have been related by one of the expressions derived by Alberty (3). It is concluded that, although a single mechanism cannot be assigned to the reaction, the most probable reaction sequence involves one or more ternary complexes of enzyme, coenzyme, and substrate.

The variation of the equilibrium constant of the reaction with lactate concentration, first observed by Kubowitz and Ott (8), has been assigned to enzymatically reducible impurities present in the lactate. The value of ΔH for the reaction, calculated from the variation of the equilibrium constant with temperature, has been found to agree well with that recently determined by direct calorimetry by Katz (28).

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THE TITRATION OF SOME PHOSPHATIDES AND RELATED COMPOUNDS IN A NON-AQUEOUS MEDIUM*

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Information regarding the ionic species of the phosphatides as they exist in lipide solvents may be inferred from their acid-base titration curves in such solvents. A number of titrations of lecithin (2–4) and of "cephalin" (2, 4, 5) have been reported. However, the chemical composition of some of the preparations employed, especially the "cephalin," is questionable. In addition, cross-comparison of the available data is difficult, as different solvents were used by the various investigators. Finally, evaluation of the behavior of the phosphatide functional groups in aqueous medium remains uncertain. The clarification of the chemical species present in brain "cephalin" by Folch (6–9) and the availability of synthetic phosphatides by the methods of Baer *et al.* (10, 11) have suggested the reexamination of the properties of the phosphatides when titrated in non-aqueous media.

An electrometric titration system in which a solvent mixture which dissolves a large number of lipides is employed has been developed. Titrations of phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, a phosphatidic acid, and fatty acids, as well as related compounds, were carried out. The method described should be of interest in connection with the quantitative determination and identification of small amounts of lipide substances. Further, the data obtained permit inferences to be drawn regarding the ionic species present in solution under conditions defined as to solvent composition, inorganic ions present, and apparent pH as measured by the glass electrode.

* This investigation was supported by a contract with the Atomic Energy Commission and by the Eugene Higgins Trust through Harvard University. Some of the data presented are from a thesis submitted by James E. Garvin to the Faculty of the Graduate School of Arts and Sciences of Harvard University, Division of Medical Sciences, in partial fulfilment of the requirements for the degree of Doctor of Philosophy. A preliminary report has already been presented (1).

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Materials

In the titrations the following materials were employed: potassium acid phthalate, Mallinckrodt primary standard; DL-alanine, Eastman No. 845 (total nitrogen, theory 15.7 per cent, found 15.8 per cent); glycine, Eastman No. 445 (total nitrogen, theory 18.7 per cent, found 19.3 per cent); DL-serine, Merck recrystallized (total nitrogen, theory 13.8 per cent, found 13.4 per cent); ethanolamine, Eastman No. 1597, distilled from potassium hydroxide just before use, b.p. 168–171° (corrected); stearic acid, Merck, five times recrystallized, m.p. 69.5–70.0°; palmitic acid, Eastman No. 1213; lauric acid, Eastman No. 933; myristic acid, Matheson No. 2431; benzoic acid, Mallinckrodt analytical reagent, sublimed two times; acetic acid, Baker's analyzed; lactic acid, Mallinckrodt 85 per cent; *p*-hydroxybenzoic acid, Poulenc Frères, resublimed, m.p. 214° (corrected); β-glycerophosphoric acid, disodium salt (pentahydrate), Eastman No. 3532, maximal content of the α isomer 0.1 per cent (total phosphorus, theory 10.1 per cent, found 9.8 per cent).

The dipalmitoyl-L-α-glycerophosphoric acid[1] was a synthetic preparation (10). The phosphatidylserine, prepared from ox brain according to Folch (8), had the following composition: N 1.76 per cent, P 3.62 per cent, NH_2-N 1.44 per cent, COOH-N[2] 1.44 per cent, nitrogen as serine 1.31 per cent, nitrogen as ethanolamine 0.03 per cent, iodine number 65. Egg lecithin prepared according to Pangborn (12) had the following composition: N 1.82 per cent, P 3.94 per cent, N:P 1.01 (molar ratio), NH_2-N less than 1 per cent (as phosphatidylethanolamine or phosphatidylserine), iodine number 54.2, soap plus free fatty acid 0 per cent. The L-α-dimyristoylcephalin, which was a synthetic preparation[1] (11), had the following composition: NH_2-N 2.1 per cent, P 4.96 per cent. The L-α-dipalmitoyllecithin[3] prepared from yeast (13) had the following composition: N 2.10 per cent, P 4.70 per cent, N:P 0.97 (molar ratio), iodine number 0, soap plus free fatty acid 8 per cent. With the exception of the rather high phosphorus and nitrogen values for the L-α-dipalmitoyllecithin, these data are in accord with those reported in the literature.

Methods

In the analysis of the materials the following procedures were employed: total phosphorus by the method of King (14); iodine number by the micromethod of Yasuda (15); total nitrogen by a wet combustion method (16) slightly modified from Uzman;[4] NH_2-N according to Van Slyke (17), as

[1] The authors are greatly indebted to Dr. Erich Baer for these preparations.

[2] We wish to express our appreciation to Dr. J. Folch and Dr. M. Lees for carrying out this determination.

[3] The authors are greatly indebted to Dr. D. J. Hanahan for this sample.

[4] Personal communication of Dr. J. Folch.

modified by Folch[4] (8) for phosphatides; nitrogen as serine or ethanolamine by the procedure of Axelrod *et al.* (18); amino nitrogen as phosphatidylserine or phosphatidylethanolamine by spot testing on paper with the buffered ninhydrin reagent of Moore and Stein (19). The free fatty acid and soap determinations were made by the non-aqueous titration procedure described in this paper.

The solvent system employed in the titrations consisted of 99 per cent (volume per volume) (11.0 M) 2-ethoxyethanol and 1 per cent (0.56 M) water. The mixture was 0.001 M with respect to KCl. This solvent mixture (without the KCl) had a dielectric constant of 14 ± 1[5] at low frequency (10^5 cycles per second). The titrations were followed by measuring the potential difference developed between a glass electrode (Beckman 4990-29) and a saturated calomel half cell reference electrode (Beckman 4970-29) immersed in the solution. Potentials, recorded in millivolts, were measured with a potentiometer (Beckman model H-2 pH meter). The materials to be titrated (approximately 1 μeq.) were dissolved in 2.0 ml. of solvent mixture and added to a small glass cup. The cup was attached to the titration apparatus with a gas-tight seal, the apparatus was flushed with nitrogen, and the titration was carried out by the addition of small increments of 0.01 N HCl or KOH in a solvent mixture of 99 per cent (volume per volume) 2-ethoxyethanol and 1 per cent water. Back-titration with either acid or base was accomplished without taking down the apparatus since two burettes, one containing acid and one base, were routinely incorporated in the arrangement of equipment. Mixing was provided by a magnetic stirrer. All the titrations were carried out at room temperature which varied from 21–27°.

In the preliminary titrations the potentiometer was standardized with 0.05 M potassium acid phthalate (pH 4.008 at 25° (20)) and Beckman buffer solution (pH 7.07 ± 0.04 at 25°). In the phosphatide titrations the following four reference solutions were used: 0.100 N HCl, pH 1.085 at 25° (21); 0.050 M potassium acid phthalate as above; phosphate buffer, pH 6.994 at 25° (22); 0.050 M sodium borate, pH 9.180 at 25° (21).

End-points and mid-points ("pK'_a")[6] were estimated from the titration curves by a simple graphical procedure, which gave greater stoichiometric accuracy and precision than the $\Delta pH/\Delta B$ method (23). A template was fitted to the titration curve plotted after subtraction of the blank and rotated through 180°. Corresponding points on the mirror image arms of the curve were noted. End-points were assigned at positions plus and minus 120 mv. (or 2.0 "pH" units) from the mid-point of symmetry.

[5] We wish to express our appreciation to Dr. J. L. Oncley and Mr. J. K. Inman for carrying out this determination.

[6] Throughout this paper apparent pH or apparent pK'_a in the 2-ethoxyethanol solvent system is referred to as "pH" or "pK'_a", respectively.

"pK′$_a$" was designated as this mid-point. "pH" numbers were assigned to the millivolt values according to the equation

$$\frac{E_s - K_b}{\Delta E_b/\Delta \mathrm{pH}_b} = \text{"pH"} \qquad (1)$$

where E_s is the measured electromotive force in the 2-ethoxyethanol solvent and $\Delta E_b/\Delta \mathrm{pH}_b$ and K_b are the slope and intercept, respectively, of the electrode pair employed with the aqueous reference standards.

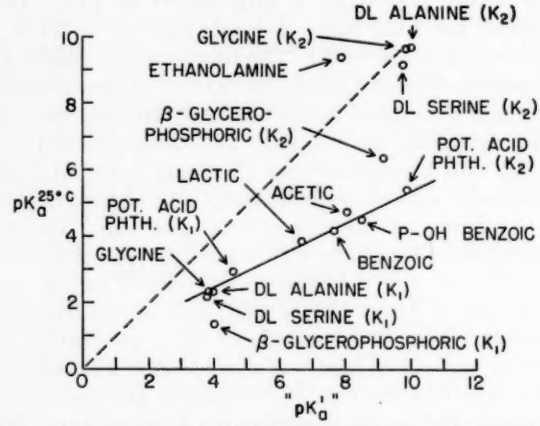

FIG. 1. Relation of apparent pK′$_a$ ("pK′$_a$") to pK$_a^{25}$ (in water). Measurement of "pK′$_a$" was made from titration curves obtained with the glass electrode and saturated calomel half cell reference electrode. The solvent system was 99 per cent (volume per volume) 2-ethoxyethanol, 1 per cent water, and 0.001 M KCl. The values for the pK$_a^{25}$ (which include a number of pK′$_a^{25}$ values) were obtained from the following sources: DL-serine, K'_1 and K'_2 (28); glycine, K'_1 and K'_2; DL-alanine, K'_1 and K'_2 (29); potassium acid phthalate, K_1 (30) and K_2 (31); lactic acid, K (32); benzoic acid, K (24); ethanolamine, K (27); acetic acid, K (24); *p*-hydroxybenzoic acid, K_1 (33); β-glycerophosphoric acid, K'_1 and K'_2 (26).

In practice when 1 to 2 μeq. was titrated, these procedures regularly gave results in the "pK′$_a$" range of 3.8 to 8.5 to within 2 per cent of the value of the carboxyl groups, as determined by independent characterization of the compounds. Thus, by using base standardized against pure benzoic acid the following purity was determined by triplicate titrations for several of the compounds noted under "Materials:" potassium acid phthalate 102 ± 1 per cent, *p*-hydroxybenzoic acid 99 ± 1 per cent, stearic acid 102 ± 1 per cent, DL-serine (carboxyl group) 98 ± 4 per cent. Outside the range of "pK′$_a$" mentioned above, the titration values were less exact. When an end-point at either margin of the titration range was

uncertain but a mid-point of symmetry could be established, the total titer was estimated by taking twice the value between the "pK'_a" and the certain end-point. This was found necessary with the 0.01 N titrants only when the end-points were less than "pH" 2 or greater than "pH" 11. An estimate of the variability of individual measurements of the "pK'_a" was provided by a comparison of the mid-points measured for the benzoic acid standard over a period of months. In thirty titrations of benzoic acid in the non-aqueous solvent mixture an average "pK'_a" of 7.79 ± 0.11 (standard deviation) was obtained. The reliability of the apparatus itself was further tested by employing it in the *aqueous* titration of representative known materials. These aqueous titrations were conducted at approxi-

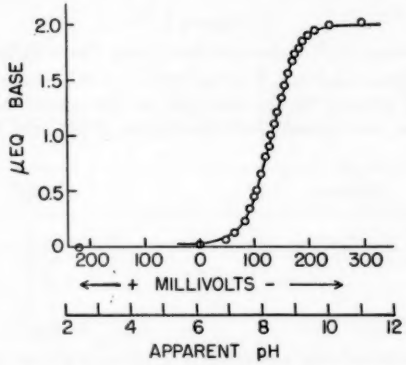

FIG. 2. Theoretical law of mass action curve compared with stearic acid titration curve. The solid line represents the mass law relationship; the circles represent the experimentally determined points after subtraction of a small blank. The solvent system was 99 per cent (volume per volume) 2-ethoxyethanol, 1 per cent water, and 0.001 M KCl.

mately 0.005 M with respect to the total amount of the compound with the following results: acetic acid, $pK'_a = 4.76 \pm 0.02$, $pK_a^{25} = 4.76$ (24); second acid dissociation constant of β-glycerophosphoric acid, $pK'_a = 6.51 \pm 0.02$, $pK_a^{25} = 6.6$ (25), and $pK'^{25}_a = 6.34$ (26); ethanolamine, $pK'_a = 9.25 \pm 0.02$, pK'^{25}_a at approximately 0.005 M = 9.36 (27).

Results

Preliminary Titration of Some Compounds Containing Carboxyl and Amino Groups Which Are Soluble in Both 2-Ethoxyethanol Solvent System and Water

The apparent pK'_a ("pK'_a") values obtained for a series of compounds were plotted against their pK_a^{25} values (in water) as shown in Fig. 1. The

solid line (obtained by the method of averages) through the points for the carboxyl groups is described by the equation

$$pK_a^{25} = 0.5\text{“}pK'_a\text{”} + 0.5 \quad (2)$$

Titration of Fatty Acids

A comparison of the experimentally obtained curve for stearic acid with the mass law equation

$$\text{“pH”} = \text{“}pK'_a\text{”} + \log \frac{[A^-]}{[HA]} \quad (3)$$

is shown in Fig. 2. The agreement is quite close. The acid dissociation

TABLE I

Calculated pK_a^{25} Values for Some Long Chain Fatty Acids

The empirical Equation 2, $pK_a^{25} = 0.5$ “pK'_a” $+ 0.5$, was used to obtain these estimates. “pK'_a” denotes the apparent pK'_a as determined in the solvent system 99 per cent (volume per volume) 2-ethoxyethanol, 1 per cent water, and 0.001 M KCl.

Fatty acid	“pK'_a”	pK_a^{25} (calculated)
Stearic acid	8.4	4.7
Palmitic acid	8.2	4.6
Myristic “	8.3	4.7
Lauric acid	8.2	4.6
Acetic “	8.1	4.6*

* Our value for acetic acid measured in the same apparatus as was used for the non-aqueous titrations was $pK'_a = 4.76$ at approximately 0.005 M. The pK_a^{25} has been determined to be 4.76 (24).

constants of five fatty acids expressed as “pK'_a” found by titration in the non-aqueous medium and as calculated pK'_a in aqueous medium from Equation 2 are shown in Table I.

Titration of Some Phosphatides

Fig. 3 and Table II show the results obtained on titrating some phosphatides (as well as DL-serine, ethanolamine, and β-glycerophosphoric acid) in the 2-ethoxyethanol solvent system. To test for a possible interaction of lecithin and fatty acid in this solvent system, 1 μmole of palmitic acid was quantitatively titrated in the presence of 5 μmoles of egg lecithin. The presence of the lecithin did not affect the “pK'_a” of the palmitic acid, nor did the presence of the palmitic acid appreciably affect the appearance of the lecithin curve outside the range of the “pK'_a” for palmitic acid ±2.0 “pH” units. The yeast lecithin preparation showed buffering correspond-

ing to 8 per cent of the phosphorus on a molar basis. The mid-point of this buffering was 8.2 ± 0.1 "pH" units. Since the amino nitrogen, as phosphatidylserine or phosphatidylethanolamine, was less than 1 per cent of the phosphorus on a molar basis, this buffering was assigned to "free" fatty acids. Of importance in this regard was the finding that the bulk of titratable fatty acid (unesterified fatty acid) was in the salt form. Hence, if the determination of "free" fatty acid had been carried out by titration with base alone, the salt (soap) present would have been undetected.

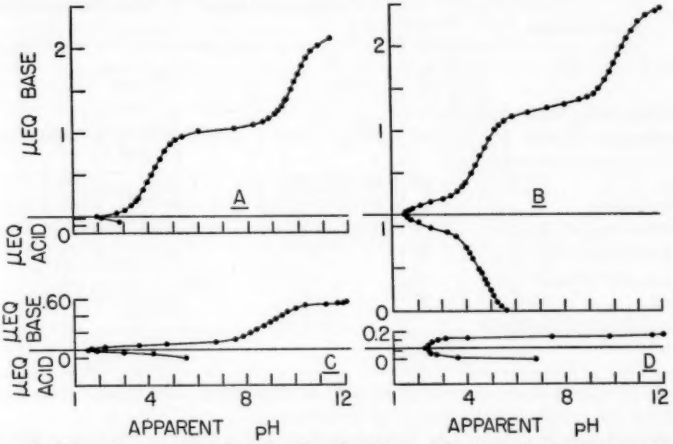

FIG. 3. Titration curves of some phosphatides. The titrations were carried out with approximately 0.01 N HCl and KOH in a solvent system consisting of 99 per cent (volume per volume) 2-ethoxyethanol, 1 per cent water, and 0.001 M KCl. The titration curves have been normalized as follows: Curve A (dipalmitoyl-L-α-glycerophosphoric acid) to 1 μeq. of phosphatide phosphorus added, Curve B (phosphatidylserine from brain) to 1 μeq. of NH_2-N and COOH-N (NH_2-N and COOH-N were exactly equal for this preparation), Curve C (L-α-dimyristoylcephalin) to 0.25 μeq. of NH_2-N added, Curve D (egg lecithin) to 5 μeq. of phosphatide phosphorus added.

DISCUSSION

The quantitative correspondence of the titrations with the value of the functional groups as determined by separate chemical analysis is taken as support for the belief that these titrations are actually of the groups indicated. Thus the contour of the titration curve and its position in the "pH" scale serve both to identify and determine quantitatively the various groups. In addition, the close conformity of the titration curves to contours consistent with simple mass law considerations suggests that the ionic species present in the solvent at the different points in the titration are such as this law predicts. Hence, although the "pH" numbers are recognized as

arbitrary, they would appear to provide information about the events during titration. The presumed charge types for a number of lipides as a function of "pH" as measured by the glass electrode are summarized in Fig. 4. No assumptions are made concerning association with anions or cations or solvent molecules.

TABLE II

*Quantitative Data from Titrations in 2-Ethoxyethanol Solvent System**

Material titrated	Amount used†	Acid-dissociable groups		Base-dissociable groups	
			"pK′$_a$"‡		"pK′$_a$"‡
	μeq.	*μeq.*		*μeq.*	
(*A*) Dipalmitoyl-L-α-glycerophosphoric acid	0.89	0.88 ± 0.01	4.1 ± 0.1	0.94 ± 0.0	10.0 ± 0.1
(*B*) β-Glycerophosphoric acid	1.00	1.03 ± 0.01	4.0 ± 0.1	0.99 ± 0.04	9.2 ± 0.1
(*C*) DL-Serine	0.91	0.89 ± 0.04	3.8 ± 0.1	0.97 ± 0.02	9.8 ± 0.1
(*D*) Phosphatidylserine	0.76	0.79 ± 0.01	4.6 ± 0.00	0.86 ± 0.03	10.3 ± 0.1
(*E*) Ethanolamine§	0.90			0.91 ± 0.01	8.1 ± 0.0
(*F*) L-α-Dimyristoylcephalin‖	0.23			0.41 ± 0.01	9.1 ± 0.1
(*G*) L-α-Dimyristoylcephalin¶	0.11			0.15 ± 0.01	9.1 ± 0.2

* All titrations were carried out at least in duplicate. The mean values are followed by the maximal deviation from the mean.

† All values were obtained by independent chemical analysis relating to the functional groups as follows: (*A*) and (*B*) by phosphorus determination, (*C*) by total nitrogen determination, (*D*) carboxyl group by COOH-N determination and α-amino nitrogen group by total NH_2-N determination, (*E*), (*F*), and (*G*) by total NH_2-N determination.

‡ "pK′$_a$" refers to the apparent pK′$_a$ obtained in the 2-ethoxyethanol solvent system in which the glass electrode and saturated calomel half cell reference electrode are employed.

§ Stirred at apparent pH ("pH") 1.8 for 1 hour.

‖ Initial titration with HCl immediately followed with KOH.

¶ Stirred for 1 hour at apparent pH ("pH") 3.5 before titration with KOH.

The direction and magnitude of the acid dissociation constant changes in the 2-ethoxyethanol solvent system as measured with the glass electrode are quite similar to what would have been expected if the hydrogen electrode had been employed. This is apparent when the values reported here are compared with those for the same compounds in alcohol (34–36). This conformity applies to carboxyl, amino, and α-amino groups.

The correlation, noted in Equation 2, for the half titration points of the carboxyl groups of the compounds soluble in both water and the 2-ethoxy-

ethanol solvent system is quite striking, but its theoretical significance is obscure. It does, however, provide an empirical index to the behavior in water solution of carboxyl groups attached to moieties rendering the molecule so water-insoluble as to make direct measurement impossible. The conclusion that the long chain, saturated fatty acids have acid dissociation constants (water) only slightly smaller than those of the shorter members of the series agrees with the data of Noerland (quoted by Schmidt-Nielsen (37)) who found little difference in the "pK'_a" values of fatty acids containing 6 to 16 carbon atoms titrated in 95 per cent ethanol.

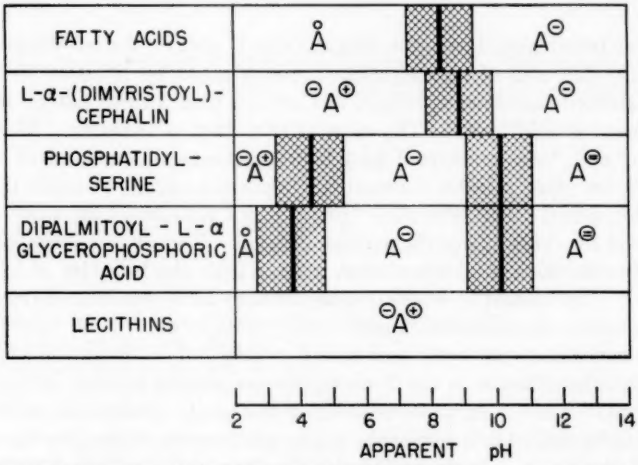

FIG. 4. The apparent pK′ and presumed ionic charge types of some lipides when dissolved in the 2-ethoxyethanol solvent system. The dark lines represent the apparent pK'_a and the cross-hatched areas the zone of 80 per cent transition of species.

The application of the correlation noted in Equation 2 to the carboxyl group of phosphatidylserine seems of interest, especially when it is noted that the non-aqueous titration curves of DL-serine and phosphatidylserine are very similar in contour. The phosphatide curve is displaced by less than 1 "pH" unit to higher values. On employing Equation 2, an estimate of $pK_a^{25} = 2.8$ is obtained for the carboxyl group of phosphatidylserine. If a similar calculation is made from the "pK'_a" of DL-serine, a value of $pK_a^{25} = 2.4$ is obtained for the carboxyl group of serine. The measured value for the latter is 2.2 (28). Assuming that the transfer to aqueous solution would affect the dissociation of the α-amino group of the phosphatidylserine to the same degree as a similar transfer does for DL-

serine, a value of $pK_a^{25} = 9.7$ (water) is obtained for the α-amino group of phosphatidylserine. Folch (9) has found that 1 mole of monovalent cation is associated with each mole of phosphatidylserine as isolated from ox brain. The titration curve shown in Fig. 3, Curve B is consistent with this and suggests that, in this solvent system at "pH" 7.0, the molecule exists with both the diester phosphoric acid group and the carboxyl group in the anionic form, while the α-amino function exists as a positive group of unit charge. No inferences can be made from these data regarding the ionic form in the dry state. In assigning ionic species to phosphatide molecules, it is important to specify the physical state to which the formulae are to apply.

It can be calculated from the data in Table II that for the L-α-dimyristoyl-cephalin the ratio of microequivalents determined by titration to microequivalents determined as NH_2-N was lowered from 1.75 to 1.36 by stirring for 1 hour at acid "pH." The cause of this effect is unknown. However, as the "pK'_a" was unaffected by 1 hour of stirring, the amount of carbon dioxide (or other possible contaminant) does not appear to affect the half titration point. The estimated "pK'_a" of 9.1 is close to the value of 8.9 obtained by Jukes (2) in 98 per cent ethanol. The pK_a^{25} is calculated to be in the direction of a higher value, judging from the behavior of ethanolamine. The amount by which it is higher may be approximated from that measured for ethanolamine, namely 1.3 pH units, or $pK_a^{25} = 10.4$ (in water). These conclusions are consistent with a zwitter ion configuration for phosphatidylethanolamine in the 2-ethoxyethanol solvent system in the range from "pH" 2 to 7. In aqueous medium one would predict the zwitter ion range to be shifted to a somewhat higher pH number at least for the upper end of the range. Again no inferences concerning the ionic form of the solid state appear warranted from these data.

The titration curve of the synthetic dipalmitoyl-L-α-glycerophosphoric acid clearly indicates that before titration it was present in solution as the dibasic acid. The titration curve of the β-glycerophosphoric acid in the 2-ethoxyethanol solvent was similar to that for the phosphatidic acid, except that the β-glycerophosphoric acid had a somewhat more acid second dissociable group (Table II). This suggests that in aqueous media the phosphatidic acid would dissociate very much like the glycerophosphoric acid. The titration curve of the egg lecithin preparation is in agreement with previous studies (2–4), indicating a zwitter ion in the neutral range both in alcoholic solvents and in water. The very basic character of the quaternary nitrogen is well shown.

SUMMARY

1. An electrometric titration method has been developed in which a solvent system consisting of 99 per cent (volume per volume) 2-ethoxyethanol,

1 per cent water, and 0.001 M KCl is used. The method has been used to titrate a variety of phosphatides and related compounds in amounts of about 1 μeq.

2. The properties of the titration curves were such as to permit assignment of ionic species present in solution for each of the materials over a wide range of apparent pH.

3. Approximations to the behavior of dissociable groups of the phosphatides in true water solution have been made by analogy with compounds having similar functional groups but which are soluble both in the 2-ethoxyethanol solvent system and in water.

We wish to express our gratitude to Professor A. B. Hastings for his interest and advice and to Professor John Edsall for valuable criticism of the manuscript.

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ENZYMATIC MECHANISM OF OXIDATION OF TARTRATE*

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Mitochondria of all animal tissues, so far investigated, contain an enzyme system which catalyzes the oxidation of *meso*- and (−)-tartrate. Certain properties of this enzyme system, such as its extraction and coenzyme requirements as well as the isolation of oxidation products of tartrate, were described in a previous publication (1). The reconstruction of Reactions[1] 1 to 3 was based on the chemical identification and rates of

(1) Tartrate + DPN^+ + Mg^{++} ⇄ DIHF (oxaloglycolate ⇄ dihydroxyfumarate) + DPNH + H^+

(2) DIHF + DPN^+ ⇄ diketosuccinate + DPNH + H^+

(3) 2DIHF + Mg^{++} → CO_2 + hydroxypyruvate; → 2 glyoxylate

formation of these compounds. A more detailed knowledge of the individual steps of the sequence of reactions is a prerequisite of further work which may lead to the isolation of the enzyme components of this system. This paper deals with experiments which were designed to fulfil this requirement.

Methods

An aqueous solution of the enzyme system, prepared from acetone powder of beef heart mitochondria (1), was used throughout these experiments. The preparation of keto acids, their isolation as 2,4-dinitrophenylhydrazine derivatives, and their separation by means of paper chromatography have

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[1] An equilibrium mixture of oxaloglycolate and dihydroxyfumarate is abbreviated in this paper as DIHF. It was previously suggested (1) that the first product of the enzymatic dehydrogenation of tartrate is the keto acid oxaloglycolate which rapidly attains equilibrium with its more stable dienol form, *i.e.* dihydroxyfumarate. The keto-enol equilibrium constant of this acid in aqueous solution is unknown. DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide, respectively; EDTA, ethylenediaminetetraacetate; *A*, absorbancy.

been described in a previous publication (1). Cytochrome *c*, DNP (90 per cent pure), DPNH, and triphosphopyridine nucleotide were obtained from the Sigma Chemical Company. All spectrophotometric measurements were carried out with the Beckman DU instrument, with 1 cm. quartz cuvettes of 1.4 ml. volume.

Results

Oxidation of Tartrate—The enzymatic reduction of DPN by tartrate (Reaction 1) was measured in 0.1 M tris(hydroxymethyl)aminomethane buffer, pH 8.3. In agreement with previous results, obtained by manometric measurements (1), the addition of Mg^{++} increased the rate of DPN reduction (Fig. 1), although the initial rates were unaffected. Under identical conditions *meso*-tartrate was more rapidly oxidized than (−)-tartrate (Fig. 2).

The nature of the O_2-consuming reaction which occurs during the aerobic oxidation of tartrate was studied in detail. A comparison of DPNH formation with O_2 absorption suggested that the reoxidation of DPNH (either by the DPNH-oxidizing system of intact mitochondria or by the non-enzymatic oxidation by phenazine methonium sulfate) could account only in part for the amount of O_2 consumed. It was, therefore, concluded that the further oxidation of DIHF must contribute to O_2 consumption.

As shown in Fig. 3, cytochrome *c* is rapidly reduced during tartrate oxidation. The enzyme preparation used in this work contained only small amounts of DPNH cytochrome *c* reductase activity; therefore, the reduction of cytochrome *c*, which occurred during the enzymatic oxidation of tartrate, was due at least to the extent of 70 per cent to a reaction between DIHF and cytochrome *c*. It was found that DIHF similar to ascorbate directly reduces cytochrome *c* at pH 8.3 in the absence of an enzyme. This reduction is inhibited by EDTA. Certain dyes, such as 2,6-dichlorophenolindophenol, can also react non-enzymatically with DIHF. The reduced form of this dye is reoxidized by O_2 only very slowly, while the reaction between reduced phenazine methonium sulfate and O_2 is rapid. This property of phenazine methonium sulfate explains its rôle as a carrier in the aerobic oxidation of tartrate. In the course of the non-enzymatic aerobic oxidation of DIHF by a suitable dye, H_2O_2 is formed. Since H_2O_2 could conceivably decompose some products of the enzymatic oxidation of tartrate, this possibility was also investigated. With 2,6-dichlorophenolindophenol or phenazine methonium sulfate as an electron acceptor, the addition of crystalline catalase to a tartrate-oxidizing enzyme reaction mixture caused only a small diminution of hydroxypyruvate formation, suggesting that the decarboxylation of DIHF was but slightly increased by H_2O_2. This effect, however, was negligible in experiments of short duration

(10 to 20 minutes). Under the experimental conditions used, catalase was not inhibited by the dyes.

Reduction of DIHF—It was reported (1) that DIHF is enzymatically reduced to tartrate when DPNH is present. As shown in Fig. 4, DPNH is rapidly oxidized by DIHF when a small amount of an extract of beef heart mitochondria is added (Reaction 1). A study of the conditions of this reaction led to the understanding of the rôle of Mg^{++} in the oxidation of tartrate. The following observations were made. A solution of DIHF

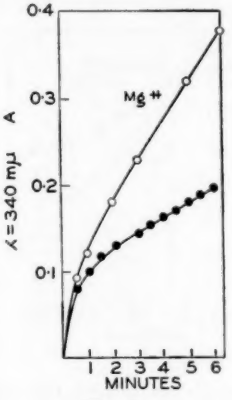

FIG. 1

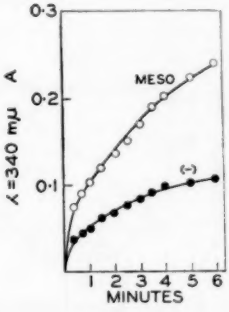

FIG. 2

FIG. 1. The effect of Mg^{++} on the enzymatic reduction of DPN by *meso*-tartrate (1.3 mg. of protein). The enzymatic reduction of DPN is measured in 0.1 M tris (hydroxymethyl)aminomethane buffer, pH 8.3, containing 1 mg. of DPN per ml. The total volume of reactants (including the soluble enzyme and 5 μmoles of Mg^{++}) is 1.4 ml. The amount of substrate is 10 μmoles.

FIG. 2. Enzymatic reduction of DPN by tartrate in the presence of Mg^{++} (0.45 mg. of protein). The conditions are the same as those in Fig. 1.

exhibits a strong absorption band at 290 mμ, characteristic of its dienol structure (determined in a range pH 4.5 to 7.0). When Mg^{++} is added to this solution, the absorbance at 290 mμ diminishes at a linear rate (approximately equivalent to 1 to 2 μmoles of dienol disappearance per hour) at room temperature. Analyses of the DIHF solution (as the 2,4-dinitrophenylhydrazone of the keto form of this acid, *i.e.* as oxaloglycolate) after several hours of incubation with Mg^{++} revealed that the oxaloglycolate content had diminished and, among other products, hydroxypyruvate had appeared. It was found that EDTA inhibits this decarboxylation catalyzed by metal ion. When Mg^{++} is added to a solution of DIHF + EDTA, a very slow diminution of the absorbance at 290 mμ occurs. However,

the oxaloglycolate content of the solution remains unchanged under these conditions, as measured by keto acid analyses (1) of samples taken at 1 minute intervals for 10 minutes. As shown in Fig. 4, preincubation of DIHF with EDTA-Mg^{++} results in an increase in the enzymatic reoxidation of DPNH.

Oxidation of DIHF—The oxidation product of DIHF is diketosuccinate. This keto acid was isolated as the 2,4-dinitrophenylosazone when the enzymatic oxidation of tartrate was allowed to proceed for a short time (10 to 20 minutes). Diketosuccinate is very unstable and is rapidly decom-

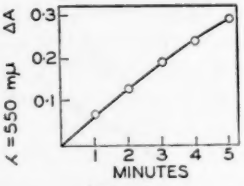

FIG. 3

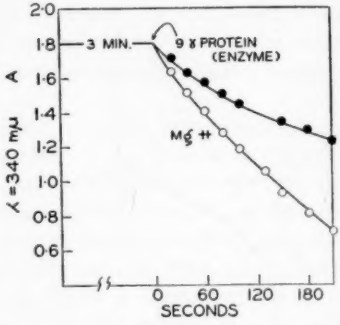

FIG. 4

FIG. 3. Reduction of cytochrome *c* during tartrate oxidation (pH 8.3, 0.45 mg. of enzyme protein). The reaction mixture contains the same constituents as described in the legend of Fig. 1 and, in addition, 1.2 mg. of cytochrome *c*.

FIG. 4. Enzymatic oxidation of DPNH by dihydroxyfumarate (pH 6.8). The enzymatic oxidation of DPNH (20 γ) is measured in 0.5 M phosphate buffer, pH 6.8, containing 4 mg. of DIHF per 5 ml. Preincubation with 5 μmoles of Mg^{++} for 3 minutes. The reaction is started by addition of the enzyme (0.01 ml.). No reaction occurs without substrates. The final volume is 1.4 ml.

posed in aqueous solution to tartronate at room temperature. EDTA slows down this decomposition. A solution of diketosuccinate + EDTA (in 0.5 M phosphate buffer, pH 6.8) is stable for 30 to 45 minutes if kept in an ice bath.

An aqueous extract of acetone powder of beef heart mitochondria does not oxidize DIHF to diketosuccinate in the presence of O_2. Similarly, the enzymatic reduction of DPN to DPNH could not be directly demonstrated with DIHF as substrate in a range from pH 7.5 to 9.0. The use of phenazine methonium sulfate or 2,6-dichlorophenolindophenol as an electron acceptor is complicated by the fact that these dyes oxidize DIHF non-enzymatically (see above). However, the DPN-linked enzymatic oxidation of DIHF (Reaction 2) can be demonstrated if correction is made for the non-

enzymatic reaction between the dye and DIHF. A solution of DIHF (4 mg. dissolved in 5 ml. of 0.1 M tris(hydroxymethyl)aminomethane buffer, pH 8.3) reduces 2,6-dichlorophenolindophenol at a rate of 34 γ per 10 minutes at room temperature. This rate is not changed when the enzyme preparation (2 mg. of protein) is added; however, the addition of DPN + enzyme increases the rate of dye reduction to 42 γ per 10 minutes. Under given conditions DPN does not react with DIHF non-enzymatically, nor does DPNH with 2,6-dichlorophenolindophenol. This observation indicates that a DPN-linked enzymatic oxidation of DIHF can be shown only when one of the products, *i.e.* DPNH, is instantly reoxidized by another

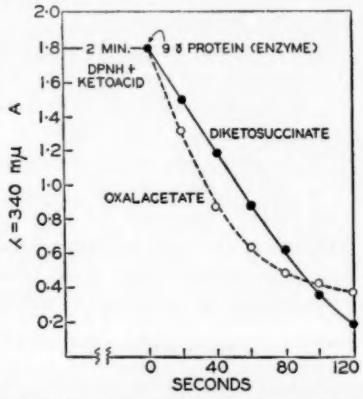

FIG. 5. Enzymatic oxidation of DPNH by diketosuccinate and oxalacetate (pH 6.8). The enzymatic oxidation of DPNH (20 γ) is measured in 0.5 M phosphate buffer, pH 6.8, containing 4 mg. of diketosuccinate or oxalacetate + 4 mg. of EDTA per 5 ml. of buffer. The conditions are the same as those in Fig. 4.

enzyme system (in this case diaphorase, present in the extract of mitochondria, which catalyzes the oxidation of DPNH by 2,6-dichlorophenolindophenol). This is in contrast to the enzymatic oxidation of tartrate by DPN which can be directly measured spectrophotometrically by the appearance of DPNH (see Figs. 1 and 2).

Triphenyltetrazolium chloride, a dye previously used for the determination of succinic dehydrogenase (2), does not react non-enzymatically with DIHF. However, this dye was not suitable for the demonstration of the DPN-linked enzymatic oxidation of DIHF. It should be mentioned that when triphenyltetrazolium chloride was used as an electron acceptor for the enzymatic oxidation of tartrate (in the presence of DPN under anaerobic conditions) the only products of the oxidation of tartrate were oxaloglycolate and glyoxylate. This reaction was increased by Mg^{++}.

Reduction of Diketosuccinate—Diketosuccinate does not oxidize DPNH non-enzymatically, but a rapid reaction occurs when a small amount of enzyme is added (Reaction 2). The product of the enzymatic reduction of diketosuccinate by DPNH is DIHF, which was identified by chromatography. The reduction of diketosuccinate under these conditions proceeds by way of DIHF partly to tartrate. As shown in Fig. 5, the enzyme preparation reduces both diketosuccinate and oxalacetate rapidly.

Other Reactions of DIHF (Reaction 3)—Hydroxypyruvate was found to be the product of an Mg^{++}-catalyzed non-enzymatic decarboxylation. This decarboxylation of the keto acid oxaloglycolate,[1] similar to that of oxalacetate as found by Krebs (3), proceeds very likely by the same mechanism described by Steinberger and Westheimer (4).

The rate of glyoxylate formation during the enzymatic oxidation of tartrate indicated that its precursor was the keto form of dihydroxyfumarate, *i.e.* oxaloglycolate (1). Incubation of DIHF with the enzyme preparation resulted in glyoxylate formation only when Mg^{++} + EDTA were added. The conversion of DIHF to glyoxylate occurred even in the absence of enzyme, provided Mg^{++} + EDTA were present. Since the rate of production of glyoxylate from DIHF, catalyzed by Mg^{++} + EDTA, was not increased upon the addition of the enzyme preparation, it was concluded that glyoxylate is formed non-enzymatically under these experimental conditions. A probable mechanism of this reaction will be discussed later.

DISCUSSION

Mitochondrial preparations catalyze the following two reactions: (*a*) the reaction of tartrate and DPN to oxaloglycolate (the keto form of dihydroxyfumaric acid) + DPNH, and (*b*) the oxidation of oxaloglycolate by DPN to diketosuccinate + DPNH. However, this second reaction can be shown only when DPNH is instantly reoxidized (*e.g.* by diaphorase + 2,-6-dichlorophenolindophenol). In the soluble enzyme system the demonstration of Reaction *b* is complicated by the fact that artificial electron acceptors such as phenazine methonium sulfate, cytochrome *c*, or 2,6-dichlorophenolindophenol oxidize DIHF non-enzymatically to diketosuccinate. In such a system DPNH produced in Reaction *a* reacts enzymatically with diketosuccinate to yield DIHF. This explains the accumulation of DIHF during the oxidation of tartrate. In intact mitochondria, in which an efficient DPNH cytochrome *c*-cytochrome oxidase system operates, the DPN-linked enzymatic oxidation of oxaloglycolate to diketosuccinate may easily occur. Further, oxaloglycolate can undergo either decarboxylation, catalyzed by Mg^{++} alone, or cleavage to glyoxylate, a reaction which occurs non-enzymatically in the presence of Mg^{++} + EDTA. Diketosuccinate is rapidly decomposed by traces of metal ions. The activating effect of Mg^{++} on the enzymatic oxidation of tartrate can be explained by the non-en-

zymatic removal of oxidation products. However, the fact that the reverse reaction, *i.e.* the reduction of DIHF to tartrate, is equally activated by Mg^{++} (in the presence of EDTA) points to a different rôle of Mg^{++}. There is reason to believe that, in the presence of Mg^{++} + EDTA, the dienol, which is preponderant in aqueous solutions (5), is more readily converted to the presumably reactive keto form (*i.e.* oxaloglycolate). This keto acid is then enzymatically reduced by DPNH. Besides the enzymatic evidence, the reactivity of DIHF with 2,4-dinitrophenylhydrazine can also be used as a proof in favor of this explanation. DIHF reacts only slowly with this carbonyl reagent and in dilute solutions it takes 10 to 12 hours (at 4°) before the crystalline derivative appears. Preincubation of DIHF with Mg^{++} + EDTA greatly accelerates the formation of the 2,4-dinitrophenylhydrazone which crystallizes in 10 to 20 minutes.

It is possible that Mg^{++} may form an additional complex with the substrate (four out of six coordination valences of Mg^{++} are occupied by EDTA while the remaining two participate in the complex with the carboxylic acid). Pedersen proposed a somewhat similar mechanism of the Cu^{++}-catalyzed bromination of acetoacetic ester (6). According to this author, the Cu^{++} complex of the enolate ion of ethyl acetoacetate plays an essential rôle in the bimolecular transfer of H^+ to a base.

Stafford, Magaldi, and Vennesland (7) reported that plant extracts contain enzymes which oxidize DPNH by DIHF and diketosuccinate. These reactions appear to be analogous to the ones catalyzed by the enzyme system of animal mitochondria. Preliminary experiments with mitochondria of lupine seedlings indicated that they also contain a tartaric acid oxidase which attacks (−)- and *meso*-tartrate. As pointed out by Stafford *et al.* (7) plants contain a DIHF oxidase, previously described by Banga and Philippot (8). Further work is required to clarify the nature of this plant enzyme.

SUMMARY

1. A soluble enzyme system, prepared from beef heart mitochondria, reduces DPN at pH 8.3 when (−)- or *meso*-tartrate is added as substrate. Mg^{++} activates this reaction. *meso*-Tartrate is oxidized more rapidly than (−)-tartrate. The oxidation product appears to be oxaloglycolate which is in equilibrium with its more stable dienol form, *i.e.* dihydroxyfumarate.

2. Dihydroxyfumarate (in equilibrium with oxaloglycolate) is enzymatically reduced to tartrate by DPNH in phosphate buffer of pH 6.8 with EDTA present. The rate of reaction is doubled when Mg^{++} is also added.

3. An equilibrium mixture of dihydroxyfumarate and oxaloglycolate is oxidized non-enzymatically to diketosuccinate by cytochrome *c*, phenazine methonium sulfate, or 2,6-dichlorophenolindophenol.

4. The enzymatic oxidation of an equilibrium mixture of dihydroxyfuma-

rate and oxaloglycolate by DPN to diketosuccinate can be demonstrated only when the reaction is coupled to a DPNH-oxidizing enzyme system (diaphorase).

5. Diketosuccinate is enzymatically reduced to oxaloglycolate by DPNH.

6. Mg^{++} catalyzes the decarboxylation of oxaloglycolate to hydroxypyruvate, while Mg^{++} + EDTA catalyze the cleavage to glyoxylate.

It is a pleasure to acknowledge the contribution of Dr. D. D. Davies whose suggestions greatly added to the development of these experiments.

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REVERSAL OF RESPIRATORY DECLINE IN NECROTIC LIVER DEGENERATION BY INTRAPORTAL TOCOPHEROLS

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Necrotic liver degeneration is produced in rats by diets deficient in vitamin E, cystine, and Factor 3 (1). During the latent phase of the disease, several weeks before the onset of acute hepatic necrosis, a defect in oxidative metabolism has been demonstrated in livers of animals on such diets. Oxygen consumption of histologically unaffected liver slices breaks down after a brief initial period of normal respiration (2). This decline of O_2 uptake is associated with an impaired utilization of acetate-C^{14} for lipogenesis, ketogenesis, and CO_2 formation (3). The agents preventing hepatic necrosis, vitamin E, cystine, or Factor 3, also forestall the respiratory lesion effectively when supplemented to the diet.

The addition *in vitro* of α-tocopherol to liver slices from deficient rats, either as an emulsion or in water-soluble form, has no significant effect on the metabolic lesion.[1] However, preliminary studies showed that injection of emulsions of *dl*-α-tocopherol into the portal vein caused the almost immediate disappearance of the defect (4). The present report describes this phenomenon; the relative potencies of several forms of α-tocopherol and of the other tocopherols (β-, γ-, and δ-) are compared. Both α- and γ-tocopherol were found to be quite potent in their ability to reverse the metabolic lesion upon intraportal injection; β- and δ-tocopherol were much less so.

EXPERIMENTAL

Male, weanling Sprague-Dawley rats of the National Institutes of Health strain, 18 to 22 days old, were fed a basal vitamin E-free diet[2] containing *Torula* yeast as the sole source of protein for periods of 24 to 59 days (body weight 60 to 120 gm.). The average survival time of rats of this strain

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[1] Unpublished experiments.

[2] The basal diet contained 30 per cent *Torula* yeast, 59 per cent sucrose, 5 per cent vitamin E-free lard, 5 per cent salts, and 1 per cent vitamin mixture (2, 5).

fed this diet is about 45 days under the experimental conditions employed here (5).

Anesthesia was induced by intravenous or intraperitoneal injection of sodium pentobarbital (2.5 mg. per 100 gm. of body weight). The abdomen of the animal was opened, and a small lobe of liver removed for the preparation of preinjection control slices. 0.2 to 0.5 ml. of emulsion at body temperature was injected directly into the portal vein and the abdomen closed. After 30 minutes the animal was sacrificed by decapitation, the liver examined for evidence of necrotic degeneration, and postinjection slices prepared. Each animal thus served as its own control.

The areas taken for slices were free of necrosis (2). Slices were prepared free-hand, blotted free of excess moisture, and weighed. Duplicate samples of approximately 100 mg. were incubated in Warburg vessels containing 3 ml. of oxygenated Krebs-Ringer-phosphate buffer (pH 7.4) with 0.01 M glucose (6). Bath temperature was maintained at 37.5° and readings were made at 10 to 20 minute intervals for 2 to 3 hours. Oxygen consumption was calculated in terms of microliters of oxygen consumed per 100 mg. of fresh weight of slices per hour of incubation (Q_{O_2} (F 100)).

Preparation of Tocopherol Solutions—A water-soluble preparation of *d*-α-tocopherol, designated as *d*-α-tocopheryl polyethylene glycol-1000 succinate, containing approximately 30 per cent, by weight, of α-tocopherol, was made up in 0.9 per cent NaCl. The free α-tocopherol employed was either the synthetic, racemic material,[3] or *d*-α-tocopherol of natural origin. The other tocopherols were the free, natural alcohols.[4] The highly viscid oils were diluted with olive oil, which contains negligible amounts of vitamin E (7). 0.5 ml. of olive oil, 100 mg. of glycerol monostearate, and 9.5 ml. of 5 per cent glucose were warmed and homogenized in a tube with a tight fitting Teflon pestle. The mixtures were homogenized before each injection.

Results

The intraportal administration of emulsions of olive oil, glycerol monostearate, and glucose, but without tocopherol, did not affect the respiratory decline (Table I). Addition to the emulsion of small amounts of α- or γ-tocopherol, or of larger amounts of β- or δ-tocopherol, resulted in sustained maintenance of oxygen uptake by the liver slices for 2 hours or longer. The presence of necrotic areas in the liver did not preclude the response to injected tocopherol by the slices, prepared from grossly and histologically

[3] The racemic α-tocopherol was purchased from Merck and Company, Rahway, New Jersey.

[4] The naturally occurring tocopherols were purchased from Distillation Products, Inc., Rochester, New York. We are indebted to Dr. P. L. Harris for the *d*-α-tocopheryl polyethylene glycol-1000 succinate.

normal parts of the organ. The rate of *initial* respiration of the postinjection slices was, in general, not significantly greater than that of the preinjection control preparations. When an apparent increase in the initial 30 minute respiration of postinjection slices was noted, it was usually found that the oxygen consumption of the preinjection control slices had declined

TABLE I

Effect of Intraportal α-Tocopherol on Respiratory Decline of Liver Slices

α-Tocopherol injected, mg.	No. of rats	No. of rats with hepatic necrosis	Q_{O_2} (F 100)*†			
			Preinjection control		30 min. postinjection	
			0–30 min.	90–120 min.	0–30 min.	90–120 min.
(1)	(2)	(3)	(4)	(5)	(6)	(7)
Olive oil emulsion‡						
0	6	4	251 ± 46	71 ± 50	225 ± 68	79 ± 65
dl-α-Tocopherol emulsion						
<0.05	8	7	224 ± 24	83 ± 32	239 ± 38	119 ± 55
0.1	12	7	225 ± 49	62 ± 48	248 ± 40	151 ± 63§
0.2	25	17	217 ± 38	66 ± 27	226 ± 51	145 ± 54§
0.4	3	2	261 ± 19	105 ± 29	310 ± 49	261 ± 29§
0.8	4	3	254 ± 40	94 ± 52	284 ± 20	258 ± 37§
>1.0	4	3	205 ± 11	70 ± 30	246 ± 61	226 ± 69§
d-α-Tocopherol emulsion						
0.1	5	1	236 ± 30	110 ± 55	285 ± 25	180 ± 35
0.2	10	2	244 ± 64	111 ± 55	290 ± 45	226 ± 63§
0.4	4	0	242 ± 59	68 ± 22	325 ± 49	267 ± 51§
d-α-Tocopherol polyethylene glycol-1000 succinate						
0.05	3	2	269 ± 56	36 ± 20	251 ± 39	113 ± 28§
0.1	8	3	232 ± 52	129 ± 67	252 ± 31	218 ± 38§
0.2	8	3	223 ± 38	100 ± 48	264 ± 39	213 ± 51§

* O_2 (c.mm.) consumed per hour per 100 mg. of fresh weight of slices, calculated for first and fourth 30 minute incubation periods.

† Mean and standard deviation.

‡ The emulsions contained 9.5 ml. of 5 per cent glucose, 100 mg. of glycerol monostearate, and 0.5 ml. of olive oil; tocopherols were dissolved in olive oil. 0.2 to 0.5 ml. of emulsions injected intraportally.

§ The differences between the values in Columns 5 and 7 are significant; $p < 0.02$.

appreciably even within this brief period. There was no evidence of a direct stimulation of respiration by the tocopherols.

dl-α-Tocopherol—In Table I the oxygen consumption of liver slices from rats on the basal diet is compared with that of slices from these same animals 30 minutes after the intraportal injection of *dl*-α-tocopherol emulsions. 56 rats received such emulsions; thirty-nine of the rats were found to have areas of hepatic necrosis. The liver appeared to be normal in the others. About 0.1 mg. of *dl*-α-tocopherol evidently altered the O_2 uptake of postinjection slices. The decline in respiration during the 2nd hour was partially prevented, as indicated by the differences between the values for the pre- and postinjection 90 to 120 minute intervals. However, doses of more than 0.4 mg. were necessary to maintain for 2 hours the respiration of postinjection slices at about their initial (30 minute) rate. Above this level, no significant differences existed between the postinjection values for 0 to 30 minutes, and 90 to 120 minutes of incubation.

d-α-Tocopherol—The data obtained with *d*-α-tocopherol prepared as an emulsion for intraportal injection compare well with those reported for the racemic mixture. A highly significant difference in the respiration of pre- and postinjection slices was observed 90 to 120 minutes after injection of 0.2 mg. of the *d*-α-tocopherol emulsion ($p < 0.01$). Whether the *d* form is more active than the *dl*-α-tocopherol cannot be concluded from these data.

Water-Soluble d-α-Tocopheryl Polyethylene Glycol-1000 Succinate—This substance was administered to nineteen animals in doses of from 0.05 to 0.2 mg. Eight of these rats displayed gross evidence of hepatic necrosis, while the liver appeared normal in the others. The results are summarized in Table I. As little as 0.05 mg. of α-tocopherol in this form significantly affected the respiratory decline of slices from deficient rats, and 0.1 mg. maintained the oxygen uptake at approximately the initial 30 minute rate for the 2 hour incubation period.[5]

d-β-Tocopherol—Fifteen rats were injected with 0.2 to 1.0 mg. of *d*-β-tocopherol in an emulsion (Table II). Twelve of the animals had gross liver damage. Administration via portal vein of less than 0.5 mg. did not affect the respiratory decline of liver slices. As much as 1.0 mg., while maintaining the oxygen consumption for somewhat longer periods, did not prevent marked decline in the rate of O_2 consumption; in six of the nine instances, the value during the 90 to 120 minute period was less than 80 per cent of the initial 30 minute Q_{O_2} (F 100).

d-γ-Tocopherol—In Table II are recorded the results obtained with thirty

[5] When added directly to the Warburg medium, as much as 0.7 mg. of α-tocopherol in the water-soluble form did not prevent the decline in respiration of liver slices from deficient rats.

rats injected intraportally with 0.1 to 3.2 mg. of *d*-γ-tocopherol in an emulsion. Eighteen of the rats showed gross necrotic degeneration at the time of the experiment. 0.2 mg. protected against respiratory decline of the liver slices and 0.8 mg. maintained respiration of postinjection liver slices approximately at their initial respiratory level for the full 2 hour incubation.

TABLE II

*Effect of Intraportal β-, γ-, and δ-Tocopherols on Respiratory Decline of Liver Slices**

Tocopherol injected, mg.	No. of rats	No. of rats with hepatic necrosis	Q_{O_2} (F 100)			
			Preinjection control		30 min. postinjection	
			0–30 min.	90–120 min.	0–30 min.	90–120 min.
(1)	(2)	(3)	(4)	(5)	(6)	(7)
β-Tocopherol						
0.2	2	2	281 ± 35	88 ± 54	237 ± 54	91 ± 75
0.5	4	3	269 ± 18	70 ± 44	300 ± 70	204 ± 84†
1.0	9	7	252 ± 30	68 ± 42	252 ± 67	181 ± 74†
γ-Tocopherol						
0.1	6	5	240 ± 65	70 ± 39	276 ± 42	110 ± 65
0.2	6	3	238 ± 29	69 ± 63	322 ± 31	246 ± 41†
0.4	6	2	238 ± 28	57 ± 31	261 ± 21	198 ± 38†
0.8	4	4	288 ± 57	162 ± 92	352 ± 56	316 ± 39†
1.6	4	1	280 ± 55	92 ± 61	296 ± 55	260 ± 37†
3.2	4	3	240 ± 56	70 ± 34	224 ± 35	198 ± 28†
δ-Tocopherol						
0.2–1.0	9	3	253 ± 43	87 ± 58	282 ± 57	133 ± 74
2.0	8	5	274 ± 25	63 ± 37	279 ± 30	245 ± 87†

* For the experimental conditions, see Table I.

† The difference between values in Columns 5 and 7 is significant; $p < 0.02$.

d-δ-Tocopherol—The seventeen rats injected intraportally with an emulsion of δ-tocopherol received doses of 0.2 to 2.0 mg. Eight of the animals had gross liver necrosis at the time of sacrifice. Injection of 1.0 mg. or less had virtually no effect on the decline of respiration, as compared with values for preinjection control slices. 2.0 mg., however, prevented the respiratory decline during the 2 hour incubation period.

Comparative Activities of Tocopherols—An evaluation of the dose-response curves derived from the data presented in Tables I and II is presented in Table III. Least square lines were fitted, connecting the per cent rever-

sion and the logarithm of the dose of tocopherol. The doses producing a 50 per cent reversion of the respiratory lesion were estimated from the computed constants of these lines. The relative activities of the various preparations tested indicate that the *d*-α-tocopheryl polyethylene glycol-1000 succinate is more than twice as active as *d*-α- or *dl*-α-tocopherol in reverting the respiratory decline of the preinjected control slices. The *d*-α-tocopherol did not differ from racemic α-tocopherol. When the activity of *dl*-α-tocopherol is taken as 100, the relative activities of the other tocopherols were as follows: *d*-γ-, 83; *d*-β-, 36; and *d*-δ-, 17.

TABLE III

Relative Activities of Various Tocopherols for 50 Per Cent Reversion of Respiratory Decline

Tocopherol tested	Dose producing 50 per cent reversion of respiratory lesion		Relative activity (*dl*-α-tocopherol = 100)
	Mean	68 per cent confidence limits	
	mg.	*mg.*	
d-α-Tocopheryl polyethylene glycol-1000 succinate	0.067	0.049–0.092	277
dl-α-Tocopherol	0.190	0.186–0.195	100
d-α-Tocopherol	0.174	0.148–0.205	109
d-γ-Tocopherol	0.230	0.154–0.344	83
d-β-Tocopherol	0.533	0.419–0.679	36
d-δ-Tocopherol	1.100	0.920–1.284	17

DISCUSSION

The development of dietary necrotic liver degeneration in the rat is prevented by the feeding of physiological amounts of tocopherol (8). A daily intake of about 0.05 mg. of synthetic α-tocopheryl acetate is required for 50 per cent protection under our experimental conditions (1). Selzer *et al.* (9) noted that the *weekly oral* administration of 0.4 mg. of *dl*-α-tocopheryl acetate, but not 0.8 mg. of γ- or δ-tocopherol, prevented hepatic necrosis in 40 to 90 per cent of rats maintained on a 10 per cent food yeast, 90 per cent corn-starch diet. When tested by the resorption-sterility method in the rat, the relative biopotencies of orally administered, natural α-, β-, γ-, and δ-tocopherols are approximately 100:40:8:1 (10). Only slightly different ratios are obtained by other bioassay methods (11–14).

Knowledge of the absorption and tissue distribution of various tocopherols is far from complete, but the available evidence indicates that the greater biopotency of oral α-tocopherol is related to a better absorption from the gastrointestinal tract (15–17). In the present study, absorption

has been bypassed by the injection of emulsions of the free tocopherols into the portal vein. It is clearly seen that as little as 0.1 to 0.2 mg. of *d*- or *dl*-α-tocopherol serves to reverse the defective metabolic situation demonstrable in liver slices immediately before injection. The *d*-α-tocopheryl polyethylene glycol-1000 succinate, when injected intraportally as a clear suspension in water, was found to be more than twice as effective as *dl*-α-tocopherol. The emulsion of the free tocopherol was much less dispersed, which may explain this difference in activity. Harris and Ludwig reported that both natural and synthetic esters of α-tocopherol, when given orally, are superior in potency to the respective free tocopherols (18).

When emulsions of different tocopherols were compared in our system, *d*-γ-tocopherol was almost as potent as α-tocopherol in the reversal of the metabolic lesion. This contrasts clearly with the observations made upon oral administration in other vitamin E assays (10). It tends to support the proposition that absorption from the intestine limits the oral biopotency of γ-tocopherol (17). β- and δ-tocopherol, on the other hand, were as ineffective in the intraportal test as they were orally.

Since the site and the mode of action of vitamin E in metabolism are yet unknown, it is not possible to discuss the difference between various tocopherols in terms of biochemical specificity. In our experiments several physicochemical factors other than chemical specificity could contribute to the observed dissimilarities. A vitamin E molecule *in transitu* (19) may be subject to oxidation and other chemical influences. It is bound to blood proteins (20) and thus may be carried off by the blood stream, whence it can be taken up by body fat or by other tissues of the vitamin E-deficient animal. Cellular permeability may also be involved. There are indications that, in our system, the action of tocopherol may take place in the mitochondria (2); the substance may have to reach this location before any effects become evident.

SUMMARY

1. The effects of intraportal injections of various tocopherols on the respiratory defect of liver slices in dietary necrotic liver degeneration were investigated. Emulsions of various tocopherols and water-soluble *d*-α-tocopheryl polyethylene glycol-1000 succinate did not markedly alter the *initial* rate of oxygen consumption, but reversed the respiratory decline, as evident from the pre- and postinjection oxygen consumption after 90 to 120 minutes of incubation. The degree of decline of oxygen consumption diminished with increasing doses of tocopherol.

2. A dose of 0.19 mg. of emulsified *dl*-α-tocopherol was required for 50 per cent reversion of the respiratory lesion. The *d*-α-tocopheryl polyethylene glycol-1000 succinate was more than twice as active. *d*-γ-Tocopherol,

upon intraportal injection, was almost as active as *d*- or *dl*-α-tocopherol in reversing the respiratory defect. For 50 per cent reversion, the relative activities were *d*-α-, 109; *d*-γ-, 83; *d*-β-, 36; and *d*-δ-, 17; these are compared to *dl*-α-tocopherol as 100.

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STUDIES ON URIC ACID AND RELATED COMPOUNDS*

II. PAPER CHROMATOGRAPHY OF SUBSTITUTED XANTHINES AND URIC ACIDS

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Methylated xanthines are converted in the animal organism into a mixture of xanthines and uric acids by two simultaneous processes: demethylation and oxidation (1–3). However, the sequence of these two reactions and the exact metabolic pathways are still unknown. A quantitative study of the metabolism of substituted xanthines has been attempted by many investigators. Thus, for instance, Buchanan, Christman, and Block (4) measured the increase, after ingestion of theophylline, in phosphotungstic acid-reducing material that was not destroyed by the action of uricase. Brodie, Axelrod, and Reichenthal (5) purified the metabolites of theophylline, which are excreted in an 18 hour sample of human urine, by countercurrent distribution and ion exchange chromatography. The procedure, although lengthy and requiring large volumes of urine, permits the identification and approximate estimation of the methylated uric acids, which appear as metabolites of theophylline. Weinfeld and Christman (6) identified the uric acids, excreted after application of caffeine or theophylline to various species, by paper chromatographic separation, but again took recourse to the method of Buchanan and coworkers (4) for quantitative determination. None of these procedures can be applied to the analysis of plasma, since the volume of blood required is too large.

Paper chromatography represents the most sensitive method for quantitative separation of members of a homologous series, but requires a reliable test for spot detection. In the xanthine group, fluorescence in ultraviolet light has been used by Markham and Smith (7) and Weisman *et al.* (8). The xanthines can also be made visible by complex formation with platinum chloride and potassium iodide (9). Michl (10) obtained fluorescent derivatives of xanthines by treatment with chlorine.

For uric acid and some of its alkyl derivatives spraying with an arsenophosphomolybdic reagent has been used by Johnson (11). Vischer and Chargaff (12) located uric acid on paper by applying mercuric nitrate first,

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followed by hydrogen sulfide treatment. Reguera and Asimov (13) used for the same purpose the combination silver nitrate-potassium chromate. The reduction of ferric to ferrous ion and the formation of the red *o*-phenanthroline complex of the latter have enabled Bode and Hübener (14) to detect 0.5 γ of uric acid. We have found this method applicable to all methylated uric acids, which contain at least one free NH— group. However, the background also reddens slowly and thus makes quantitative determination difficult. The reaction is negative with xanthines.

We have tried the methods mentioned above, but none of them proved useful for all derivatives under consideration. Color intensity varies in most cases from one homologue to the other and thus not only would require individual standardization, but also would set a different limit to the quantitative determination of each member of a series. The multitude of reagents, described in the literature, merely indicates the difficulties encountered in the identification of xanthines and uric acids in chromatograms. None of the color reactions can unambiguously detect quantities below about 20 to 50 γ per sq. cm.[1] It is, therefore, apparent that the most accurate and reliable method is ultraviolet spectrophotometry, whereas any colorimetric reaction may serve only for qualitative spot detection.

The present investigation has led to a simple procedure for the quantitative separation of all components of mixtures of xanthines and uric acids, such as occur in plasma and urine. A new method has been found for spot staining, which permits easy and unambiguous determination of R_F values. The ultimate analysis, however, is carried out with the unstained materials, which are extracted from the paper and measured spectrophotometrically. Only a few micrograms of each component are required; hence the method is suitable for metabolic studies.

Materials and Methods

7-Methyl- and 1,7-dimethylxanthines were obtained through the courtesy of Dr. J. J. Fox of the Sloan-Kettering Institute for Cancer Research. 3-Methyl- and 1,3-diethylxanthines were a gift of Dr. V. Papesch of G. D. Searle and Company, Chicago. 1-Methyl- and 1,7-dimethyluric acids were kindly contributed by Professor D. Keilin, Molteno Institute, Cambridge, England, and 1,3,7,9-tetramethyluric acid by Professor E. Boyland. The other derivatives were synthesized, as described in earlier papers (16, 17). 1-Methylxanthine is difficult to obtain in pure form (18). The following synthesis is based on the method of Bredereck *et al.* (19):

[1] With special ultraviolet filters, it is possible to detect purines in quantities of 1 γ per ml. or even less (15). However, the method is much less sensitive in the case of substituted uric acids.

1-Methyluric acid (25 mg.) was added to 0.4 ml. of boiling formamide. After ¼ hour the suspension suddenly became clear. Boiling was continued for another 10 minutes; the mixture was cooled and diluted with 10 ml. of water. The solution is acidified with 1 ml. of 10 per cent HCl and passed through Amberlite IR-112-H (column length 15 cm., diameter 6 mm.). After washing the column with 5 ml. of 1 per cent HCl, the adsorbed material was eluted with 60 ml. of 6 per cent ammonia and the eluate was evaporated to dryness. The residue was dissolved in 5 ml. of hot 1 per cent ammonia and decolorized with charcoal and the filtrate was again evaporated to dryness. In this way 17.5 mg. of colorless crystals were obtained. The purity of this preparation was checked by chromatographic and spectrophotometric analyses.

The reagents used for spraying are as follows: (*a*) mercuric acetate, 0.25 per cent in 95 per cent alcohol, with addition of a few drops of glacial acetic acid to prevent precipitation of mercuric oxide, and (*b*) diphenylcarbazone, 0.05 per cent in 95 per cent ethanol. Whatman paper No. 1 was used in all experiments. All solvent mixtures were prepared from commercial products. R_F values were determined on descending chromatograms. For spot detection under ultraviolet light a Magnaflux "black light" lamp was used.

Results

Detection of Xanthines and Uric Acids on Paper Chromatograms

In the method of Vischer and Chargaff (12) the paper is first sprayed with mercuric nitrate, the excess reagents are removed by washing, and the spots are then made visible by blackening with hydrogen sulfide. Since the mercuric complexes of the methylated derivatives are soluble in acid media (Bergmann and Dikstein (20)), they are easily removed by the washing step, especially if present in microgram quantities only. However, the mercuric complexes of all xanthines and uric acids are practically insoluble in *organic* liquids. We, therefore, turned to the use of alcoholic solutions for spraying; *e.g.*, spraying with mercuric acetate in acidulated ethanol, *without* subsequent washing, permits location of the complexes. Under ultraviolet light they show light blue fluorescence on a dark violet background. However, the amounts necessary for unambiguous identification of a spot of about 1 sq. cm. are in the order of 20 to 50 γ. Thus this procedure, like the "direct" method of Markham and Smith (7), is not sufficiently sensitive. In addition, it was found that ultraviolet irradiation decomposes the mercuric complexes slowly.

Because of the limited detectability in ultraviolet light of the mercuric complexes, we tried to make them visible with some of the color reagents used in inorganic analysis. Ionic mercuric derivatives, *e.g.*, give a blue-

TABLE I

Properties of Mercuric-DPC Complexes of Xanthines and Uric Acids

Substance	Heat stability of complex			Lower limit of detectability	Color of complex*	
	Time required for complete fading at				Solvent A	Solvent B or C
	90°	120°	150°			
			sec.	*γ per sq. cm.*		
None (background)	20 min.	60 sec.	15			
Uric acid	No fading after 20 min.	Strong fading after 20 min.	160	1	Violet-red	Red
1-Methyl-	Same	Strong fading after 8.5 min.	160	1	Yellow-red	Yellowish blue
3-Methyl-	"	Same	160	1	Blue	
7-Methyl-	"	"	160	1	Violet-red	Yellow-red
1,3-Dimethyl-	"	No fading after 20 min.	450	1	Violet	Violet
3,7-Dimethyl-	"	No fading after 8.5 min.	160	1	"	"
1,7-Dimethyl-	"	Same	160	1	Yellowish red	Yellow
1,3,7-Trimethyl-	Very strong fading after 20 min.	110 sec.	20	2.5	Violet	Violet
3,7,9-Trimethyl-	Very strong fading after 10 min.	150 "	25	2	"	"
1,3-Diethyl-	No fading after 20 min.	No fading after 20 min.	450	1	"	"
Xanthine	Same	Strong fading after 20 min.	160	2	"	Red-violet
3-Methyl-	"	5.5 min.	110	2	Reddish violet	"
7-Methyl-	"	No fading after 20 min.	450	2	Blue	Blue
1,3-Dimethyl-	"	200 sec.	25	5	"	"
3,7-Dimethyl-	"	260 "	30	2	"	"
1,7-Dimethyl-	"	210 "	30	2	"	"
1,3-Diethyl-	"	200 "	25	5	"	"

* The color reported refers to the freshly stained chromatograms, after air drying, but before the background has been bleached by heating.

violet color with diphenylcarbazone (DPC), whereas covalent compounds, such as mercuric chloride, do not stain with the reagent. When solid mercuric urate is immersed in an alcoholic solution of DPC, the surface of

TABLE II

R_F Values of Xanthines and Uric Acids

	R_F values in various solvents*		
	Solvent A†	Solvent B‡	Solvent C§
Xanthine	0.29	0.57	0.44
1-Methyl-	0.49		0.64
3-Methyl-	0.44	0.66	0.71
7-Methyl-	0.44	0.65	0.59
1,3-Dimethyl-	0.64	0.80	0.83
1,7-Dimethyl-	0.64	0.79	0.71
3,7-Dimethyl-	0.58	0.73	0.85
1,3,7-Trimethyl-‖	0.74	0.80	0.88
1,3-Diethyl-	0.84	0.92	0.90
Uric acid	0.23	0.31	0.28
1-Methyl-	0.38	0.53	0.51
3-Methyl-	0.32	0.52	0.53
7-Methyl-	0.35	0.44	0.46
1,3-Dimethyl-	0.52	0.69	0.63
1,7-Dimethyl-	0.50	0.62	0.64
3,7-Dimethyl-	0.48	0.65	0.67
1,3,7-Trimethyl-	0.68	0.80	0.76
1,3-Diethyl-	0.78	0.87	0.80
1,3,7,9-Tetramethyl-‖		0.86	0.80

* The R_F values were measured in descending chromatograms, after the solvent front had advanced for about 30 cm. The chromatography chambers were kept at about 27°.

† Solvent A, 95 per cent ethanol 85 ml., acetic acid 5 ml., water 10 ml.

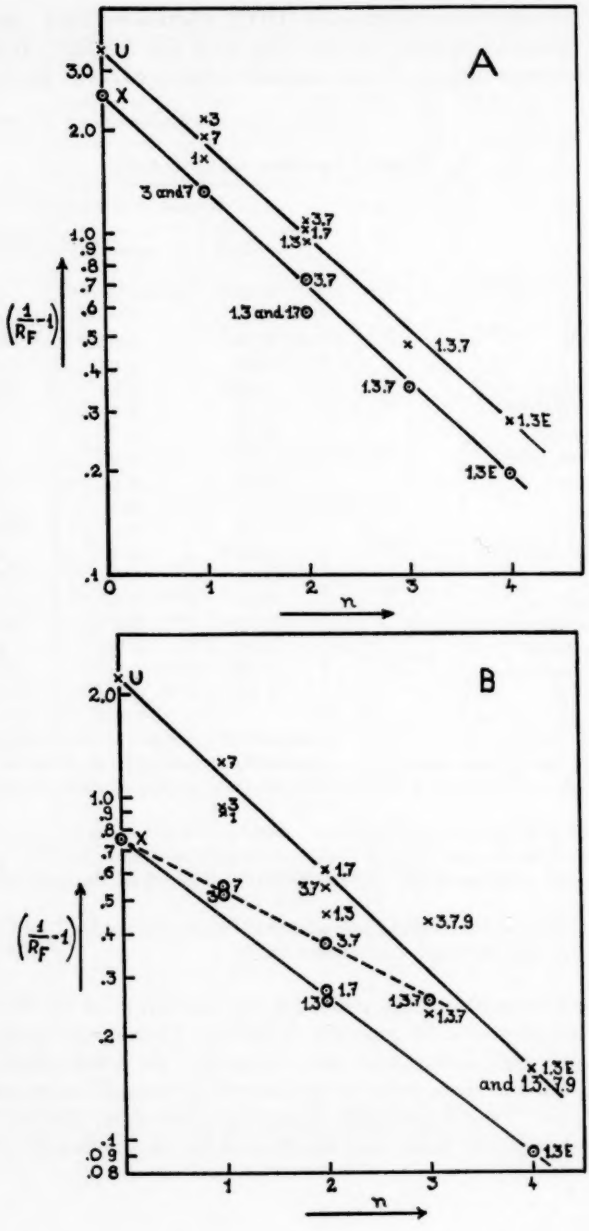
‡ Solvent B, 95 per cent ethanol 70 ml., pyridine 20 ml., water 10 ml.

§ Solvent C, pyridine 94 ml., 25 per cent NH_3— 6 ml.; final ammonia concentration, 1.5 per cent.

‖ Since caffeine and tetramethyluric acid do not stain with the Hg^{++}-DPC reagent, their positions were determined spectroscopically.

the particles stains blue-violet, indicating the ionic nature of this derivative. This method proved to be generally applicable: Upon spraying the paper first with mercuric acetate and then with DPC, the whole sheet turned red-violet, but the spots could be recognized by their different and more intense shade. Upon heating the paper over a hot-plate, the color of the background gradually faded, and the spots of the xanthines and uric acids

XUM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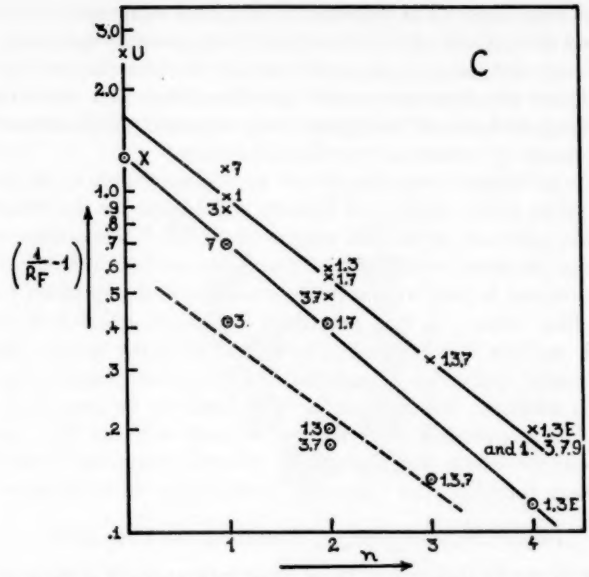

FIG. 1. The relationship between log $(1/R_F - 1)$ and n, the number of carbon atoms in the alkyl groups attached to the heterocyclic nucleus. ×, methylated uric acids (U); ⊙, methylated xanthines (X). 1,3-E indicates the diethyl derivative of the homologous series. A, Solvent A (see Table II), note the relatively small deviations from the straight lines both in the U and X series. B, Solvent B, more pronounced deviations appear in the U series. In the X series the experimental points can be represented only by two lines with a different slope. C, Solvent C, the xanthines are again represented here by two straight lines, but in this case with an identical slope.

stood out as well defined areas. Upon continued heating, these also disappeared. It was, therefore, necessary to determine the heat stability of the various mercuric derivatives. This was achieved in the following way: 10 γ of each material were put on spots of about 1 sq. cm. area, stained by the above procedure, and the time required to bleach the color at various temperatures was determined by putting the paper into a constant temperature oven. The results of these experiments are summarized in Table I. In the final procedure the paper is always heated to 90° for about 15 minutes. The exact time is not critical under these conditions. Direct heating over a hot-plate, under eye control, is a simplified modification of this procedure. The stained spots preserve their color for several months.

We now were in a position to study the lower limit of detectability for each individual derivative. The results, which are included in Table I, indicate that the procedure is suitable for the identification of microgram

XUM

quantities, such as are to be expected in biological experiments. The fully methylated derivatives, caffeine and 1,3,7,9-tetramethyluric acid, do not form mercury complexes, apparently because of the lack of a free NH—group. These two compounds were therefore located in chromatograms by extracting each cm. of the paper strip separately and measuring the amount present by ultraviolet spectrophotometry.

The only colorimetric reaction, which proved applicable to all xanthines and uric acids, is the method of Reindel and Hoppe for the detection of amino acids, peptides, and related compounds (21). These authors exposed the paper to the gases, produced from permanganate and hydrochloric acid, and then treated it with a mixture of *o*-tolidine and potassium iodide to obtain a blue color. In this procedure a positive reaction is obtained even with caffeine and tetramethyluric acid (5 γ per spot). However, with such small quantities of material, the color disappears within 1 to 2 hours. In addition, this method is very sensitive to impurities of the paper and is inapplicable after the use of basic solvents (*e.g.* pyridine), which cause the whole paper to react. For the purposes of the present investigation, therefore, the "mercury" method proved to be more useful.

Paper Chromatography of Xanthines and Uric Acids

In view of the limited solubility of these substances in organic solvents, aqueous mixtures appeared most promising for the development. The butanol-formic acid mixture, used previously by Markham and Smith (7), was tried first. It suffers from the disadvantage that its components react slowly with each other and that the increasing percentage of butyl formate changes the results from 1 day to the other. When the final equilibrium mixture was used, separation of the homologues under investigation was unsatisfactory; therefore, various mixtures with acetic acid or pyridine were studied. The R_F values of three selected solvents are summarized in Table II.

Fig. 1, *A* to *C*, demonstrates the varying separating power of three solvent mixtures. For xanthines two-dimensional chromatography is required for the analysis of an unknown mixture. In general, the R_F values of isomers are closely related and well spaced from the next higher and lower homologue. Therefore, a plot of log $(1/R_F - 1)$ *versus* n, the number of carbons in the side chains attached to the heterocyclic nucleus, gives in most cases the linear relationship postulated by Martin (22).[2]

Attempts were made to modify the chromatographic procedure in such a way as to be able to observe the migration of the various components with the naked eye. For this purpose, mercuric acetate was applied di-

[2] It should be noted that the R_F values change with the distance of travel. The values in Table II were all obtained with a solvent front about 30 cm. from the origin.

rectly to the original spot and DPC dissolved in the developing mixture. The R_F values were identical with those obtained in the absence of mercury, indicating that the solvent extracts the organic molecules and the metal ions separately and independently. It was accordingly observed that during "mercuric chromatography" the color of the advancing spots faded gradually.

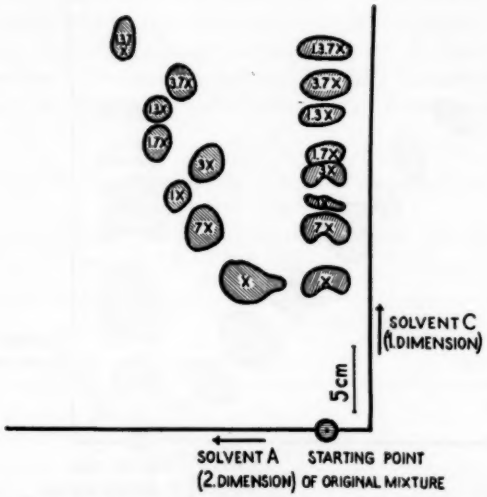

FIG. 2. Two-dimensional paper chromatogram of methylated xanthines. Location of the homologous xanthines after application of Solvent C is shown on the right side. Although 3-methyl- and 1,7-dimethylxanthines are closely connected, the two spots are distinguishable by the different color of their complexes with mercury and diphenylcarbazone (see Table I). Since these two derivatives are extracted together from the paper, it is convenient to run the chromatogram in a second dimension with Solvent A. However, it is also possible to determine a *mixture* of these two homologues spectrophotometrically, since their absorption maxima are entirely different (to be published).

Separation Procedure

Caffeine, the most highly substituted xanthine, ingested with normal food, may give a mixture of fourteen methylated xanthines and uric acids. Successful separation and determination of all possible metabolites require, therefore, first separation of the two main groups. A suitable procedure can be based on the observation that only xanthines form cations in strongly acid solutions (16). They are therefore adsorbed by a cation exchanger, such as Amberlite IR-112-H, and can be subsequently eluted by ammonia with recovery of at least 95 per cent. Similar procedures have been used

previously by Johnson (11) and Weisman, Bromberg, and Gutman (8). The separation of the members of either the xanthine or uric acid series is shown in Figs. 2 and 3. For the former group we use first Solvent C (pyridine-ammonia) and in the second direction, Solvent A (ethanol-acetic acid).[3] For the separation of uric acids Solvent B (ethanol-pyridine) has been applied in both directions. Since the ratio of the distances, covered

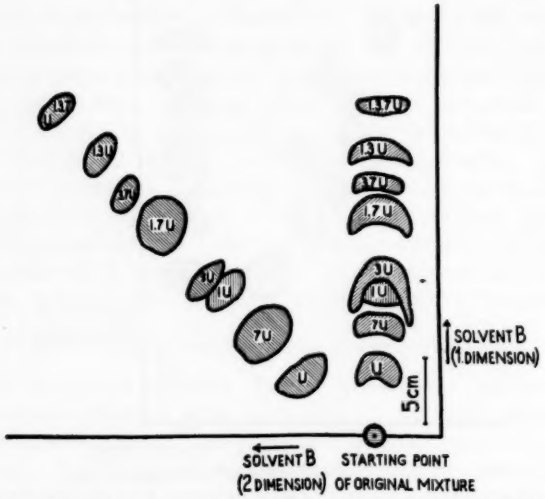

FIG. 3. Two-dimensional paper chromatogram of methylated uric acids. In both directions Solvent B was used. Therefore the spots, obtained in the first direction (at the right-hand side), as well as those in the second dimension, are situated along a straight line. The spots of 1- and 3-methyluric acids lie close together, but can be clearly distinguished because of the different shade of their Hg^{++}-DPC complexes (see Table I). For quantitative analysis these two isomers are extracted together from the paper, but do not interfere with each other in the spectrophotometric determination, since the pH dependence of their maximal extinction is entirely different (to be published).

by any individual component in the two perpendicular directions, is constant, it is to be expected that all spots are situated along a straight line. This is borne out by the experiment shown in Fig. 3. It should be noted that the solvent combinations were selected after a large number of trials and do not represent the only possible solution of the present problem.

[3] If adenine, guanine, and hypoxanthine are also present, they are adsorbed as cations and thus accompany the xanthines. In this case, the above procedure has to be modified, as will be described in the application of the method to biological fluids.

Thus, xanthines have been separated by Weisman *et al.* (8), using butanol-ammonia first and subsequently butanol-formic acid.

DISCUSSION

The curves in Fig. 1, *A* to *C*, reveal certain interesting relationships. While in Solvents A and C the straight lines for xanthines and uric acids are approximately parallel, this is not the case for Solvent B. Therefore, in both Solvents A and C, the rule of Martin (22) is obeyed; *i.e.*, for each additional CH_2— group the same increment in ΔF is obtained within a homologous series. However, in Solvent B, $\Delta\Delta F$ (increment in ΔF) decreases sharply for xanthines, while remaining at the same level for uric

TABLE III

Free Energy of Transfer of Methylene Groups Between Stationary and Mobile Phases

The figures were calculated from the slopes of the straight lines in Fig. 1, *A* to *C*, by using the equation $\Delta\Delta F = 2.3\ RT \log (1/R_{F_n} - 1)/(1/R_{F_{n-1}} - 1)$. $T = 300°$ absolute.

Solvent	$\Delta\Delta F_{-CH_2-}$ (calories)*	
	Xanthines	Uric acids
A	−390	−390
B	Upper line, −210 Lower " −320	−395
C	Upper line, −350 Lower " −350	−350

* It should be noted that the $\Delta\Delta F$ values are of the same magnitude as those obtained for the transfer of a methylene group in cholinesterase inhibitors from the free solution to the enzyme surface (23).

acids. The values of $\Delta\Delta F$, derived from Fig. 1, *A* to *C*, are given in Table III. It is concluded that in Solvent B the distribution of xanthines between the stationary and mobile phases involves an additional factor, which is of no importance for uric acids. A discussion of this problem will be given later.

All compounds included in the present investigation combine with mercuric ions, with the exception of the fully methylated derivatives, caffeine, and 1,3,7,9-tetramethyluric acid. However, the nuances of the color, formed by staining the mercuric complexes with DPC, vary from one substance to the other (Table I). It is thus indicated that the position of NH— groups, participating in complex formation, determines the specific structure and stability of the coordinative compound. Our results suggest that conjugation of an NH— group with another unsaturated structure,

e.g. C=O, is the minimal requirement for the formation of stable mercuric complexes. However, in testing this assumption, it was found that simple amides, amidines, or guanidines do not give a positive reaction. Only biuret can be stained with Hg^{++} and DPC. It thus appears that an NH— group has to be cross-conjugated with at least two unsaturated groups in order to give a positive test. All heterocyclic compounds studied so far follow this general rule. The procedure, developed in this investigation, should, therefore, be applicable to a large number of compounds, including pyrimidines, barbiturates, purine derivatives, and imidazoles. The extension of the method to these heterocycles is now under study, and the results obtained will be reported later.

The thermal stability of the complexes with Hg^{++} and DPC shows an interesting relation to structure. In the xanthine series all dimethyl derivatives fade at about the same high rate, then follows 3-methylxanthine, thereafter xanthine itself, while 7-methylxanthine is the most stable one. Evidently, when positions 1 and 3 are free, the complex is more heat-stable than when positions 1 and 7 are unsubstituted.

In the uric acid series the most stable complexes are obtained when only positions 7 and 9 are free, since both 1,3-dimethyl- and diethyluric acids excel the mother substance in their heat stability. On the other hand, if all nitrogens are occupied, besides N-1 or N-9, very unstable complexes result. This may serve as an indication that the heavy metal atom attaches itself to at least 2 heteroatoms in the uric acid structure. Therefore, the intramolecular distance between the free nitrogens determines the heat stability of the complex. However, this does not explain why the mercuric complex of 1,3-dialkyluric acids should be more stable than that of uric acid itself. Probably, free NH— groups in positions 1 and 3 compete with N-7 and N-9 for the metal and thus decrease the over-all stability of the complex. Therefore, the observed order of heat stability is 1,3-dimethyl > 1- or 3-methyluric acid or uric acid itself.

The application of the chromatographic procedure, described in this paper, to biological fluids will be reported in a future communication.

SUMMARY

Microgram quantities of xanthines and uric acids can be detected on paper by staining their mercuric complexes with diphenylcarbazone. This method is also applicable to related heterocyclic systems.

A two-dimensional paper chromatographic procedure has been developed for the separation of homologues within each series. Xanthines are first quantitatively adsorbed from a strongly acid solution onto a cation exchanger, Amberlite IR-112-H, and quantitatively eluted with ammonia before spotting. The uric acids, which pass through the column, are chromatographed separately.

A plot of log $(1/R_F - 1)$ *versus* n, the number of CH_2— groups attached to the ring system of *uric acid*, gives straight lines with almost identical slopes for various solvents. However, for *xanthines* the slope varies from one solvent to the other, and in some cases two straight lines, rather than one, represent the above function for various methylated derivatives.

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THE ISOLATION AND PROPERTIES OF MICROSOMAL CYTOCHROME*

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Mammalian liver contains a heme protein which from its spectrum and properties in particulate preparations has been termed cytochrome b_1 by Yoshikawa (1), cytochrome *m* by C. F. Strittmatter and Ball, who localized it in microsomes (2, 3), and cytochrome b_5 by Chance and Williams (4). In view of the fact that this and other microsomal enzymes have been studied chiefly in particulate suspensions, methods of isolating microsomal proteins for more detailed characterization were examined. The first and most easily recognizable of the enzymes that we have obtained is the microsomal cytochrome. This paper deals with the liberation of the cytochrome from its particulate complex, the fractionation steps in its purification, electrophoretic and ultracentrifugal tests for homogeneity, molecular weight determination, spectral properties, and reactions with a number of reagents.

Materials and Methods

Optical measurements were made in a Beckman DU spectrophotometer calibrated against emission bands of a mercury arc. Silica cuvettes of 1 ml. capacity and 1 cm. light path were used in the aerobic experiments, while, in experiments involving removal of oxygen or equilibration with another gas, cuvettes of the Thunberg type or the injection systems described in the following paper (5) were employed. Anaerobic conditions, in which no appreciable reoxidation of the cytochrome or reducing agents occurred in several hours, were obtained in such systems by alternately evacuating and flushing ten to twenty times with Linde nitrogen, and by using only solutions deoxygenated by bubbling Linde nitrogen through them for 30 minutes or longer.

Reduced di- and triphosphopyridine nucleotides (DPNH and TPNH, respectively), horse and beef heart preparations of cytochrome *c*, and recrystallized tris(hydroxymethyl)aminomethane (Tris buffers) were products of the Sigma Chemical Company. The Versene brand of ethylenediaminetetraacetic acid was employed. The indigo dyes were prepared by Dr. P. W. Preisler by the method of Sullivan, Cohen, and Clark (6).

* This work was aided by a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council.

XUM

EXPERIMENTAL

Preliminary Experiments

Microsomal fractions isolated from rat, guinea pig, rabbit, and beef livers exhibited absorption spectra in deoxycholate-clarified suspensions that were very similar to those that have been described for rat liver preparations (3). Rabbit liver was selected as a favorable compromise with respect to size and the quantity of material that could be obtained. The method of Schneider (7) for isolating the microsomes, homogenization in 0.25 M sucrose and differential centrifugation, was modified by centrifugally removing cellular débris, nuclei, and mitochondria in one step. This entailed a loss in microsomes which was more than compensated for by the gain in speed of processing large amounts of material. The factors which limited the scale of operations were the number and capacity of the high speed centrifuges. It was necessary to work batchwise with the livers of two rabbits at a time. The duration and force of the centrifugation employed in separating the microsomes were greatly diminished by addition of ammonium sulfate to 0.4 saturation. This aggregated the microsomes and at the same time precipitated very little of the soluble liver protein. One washing with dilute buffer in the preparative ultracentrifuge was employed to remove the major portion of this contaminant.

In a search of methods for isolating the microsomal proteins, acetone powders, butanol extraction, and treatment with ribonuclease, trypsin, and pancreatic lipase, both individually and in combination, were tried. Of these, only butanol extraction by methods similar to those of Morton (8) and lipase treatment yielded soluble preparations which contained the cytochrome, and by far the most consistent and successful preparations were obtained with pancreatic lipase. Incubation with lipase is used in Step 4 of the preparation. The pancreatic lipase, preliminary samples of which were supplied by Dr. R. K. Crane, was partially purified by fractional precipitation and adsorption steps (9). Since addition of trypsin inhibitor to several lipase incubations did not inhibit liberation and since trypsin itself did not liberate the cytochrome, this type of proteolysis, at least, is not involved. Also, the microsome suspensions undergo no appreciable autolysis on incubation at 37° for 1 hour, as measured by increase in ninhydrin-positive material. The lipase preparations, however, do contain a detectable amount of undefined proteolytic activity.

Preparation of Microsomal Cytochrome from Rabbit Liver

Materials—A sucrose medium, containing 0.25 M sucrose and 0.001 M Versene, pH 7.5, in glass-distilled water, is prepared in advance and prechilled in the cold room. All solid ammonium sulfate used in the prepara-

tion is purified as follows: A saturated solution is prepared with reagent grade material. Ammonia is added to approximately pH 8 and the solution is filtered. The filtrate, after addition of Versene to a concentration of 0.001 M, is concentrated by boiling and cooled to obtain crystals which are washed with cold glass-distilled water and dried at 100°. Saturated solutions of ammonium sulfate used in the preparations contained 720 gm. of reagent grade ammonium sulfate per liter of water and 3.8 ml. of concentrated ammonium hydroxide per liter of solution. After filtration, Versene is added to 0.001 M, the pH is adjusted to 7.5 with 1 N hydrochloric acid, and the solution is chilled. Thirty rabbits, weighing 5 to 7 pounds each, are sacrificed two at a time. All subsequent steps are carried out at 4–8° unless otherwise specified.

Step 1—Two rabbits are stunned and bled, and the livers excised and washed in cold sucrose media. After blotting and weighing, the livers (about 150 gm.) are minced well with scissors for 1 minute and homogenized for 20 seconds in a Waring blendor at high speed with approximately 3 volumes of sucrose media per weight of tissue. The homogenate is then diluted with sucrose media to 7 volumes per weight of tissue and is centrifuged immediately in Servall centrifuges at 80 to 82 volts (9000 × *g*) for 12 minutes. This procedure is repeated with successive pairs of animals. The total elapsed time for each pair of two rabbits is not more than 20 to 25 minutes. The volume of pooled supernatant fluid, containing the microsomes and largely free of nuclei and mitochondria, is approximately 16 liters.

Step 2—560 ml. of saturated ammonium sulfate solution are added to 800 ml. aliquots of the supernatant fluid from Step 1. After 5 minutes the suspension is centrifuged for 7 minutes in Servall centrifuges at 110 volts (11,000 × *g*). The pellets, containing the microsomes, are resuspended in a small amount of Tris buffer, 0.1 M, pH 7.5. The total volume, on resuspending the pellets from the entire 16 liters of supernatant fluid from Step 1, should be approximately 1600 ml.

Step 3—The 1600 ml. of concentrated microsome suspension are centrifuged in the No. 30 rotor of a Spinco model L ultracentrifuge at 0° for 70 minutes at 30,000 r.p.m. (80,000 × *g*). The dark red pellets are resuspended in cold 0.1 M Tris buffer, pH 7.5, to give a total volume of 1000 ml. The elapsed time up to this point is 11 to 12 hours, and the suspension may be allowed to stand overnight at 5°.

Step 4—100 ml. of the dialyzed lipase solution (9) are added to 1000 ml. of the suspension, and the mixture is gently stirred for 1 hour at 37° and then cooled in an ice salt bath to 5°.

Step 5—Ammonium sulfate fractionation of the lipase-treated preparation is carried out as indicated in Diagram 1.

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Step 6—The microsomal cytochrome preparation from Step 5 is refractionated at least two times by the procedure indicated in Diagram 2. The cytochrome obtained after the second reprecipitation, usually about 50 to

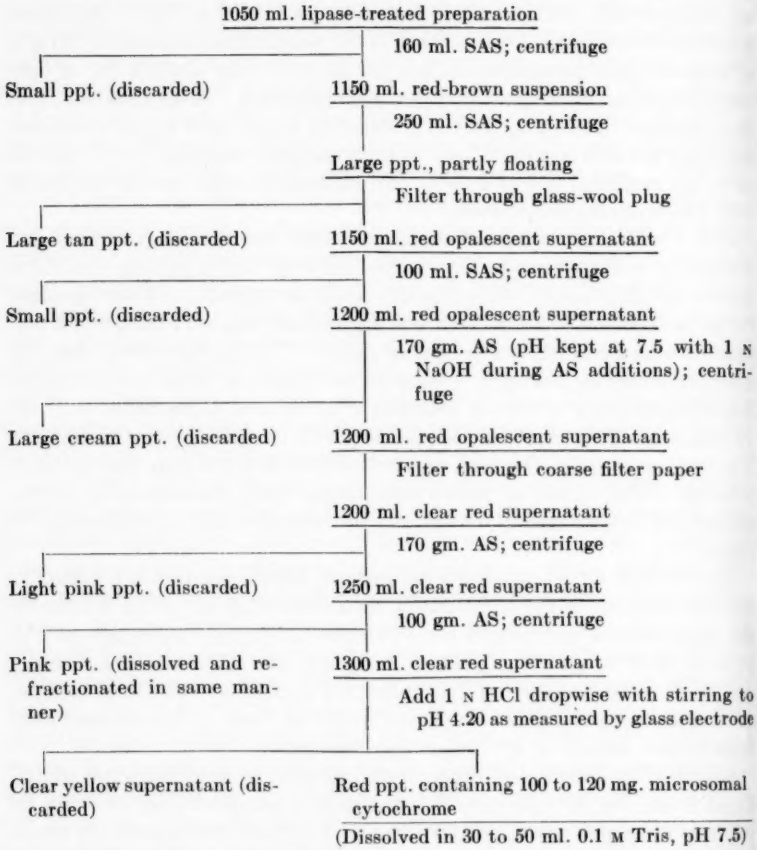
1050 ml. lipase-treated preparation
160 ml. SAS; centrifuge
Small ppt. (discarded)
1150 ml. red-brown suspension
250 ml. SAS; centrifuge
Large ppt., partly floating
Filter through glass-wool plug
Large tan ppt. (discarded)
1150 ml. red opalescent supernatant
100 ml. SAS; centrifuge
Small ppt. (discarded)
1200 ml. red opalescent supernatant
170 gm. AS (pH kept at 7.5 with 1 N NaOH during AS additions); centrifuge
Large cream ppt. (discarded)
1200 ml. red opalescent supernatant
Filter through coarse filter paper
1200 ml. clear red supernatant
170 gm. AS; centrifuge
Light pink ppt. (discarded)
1250 ml. clear red supernatant
100 gm. AS; centrifuge
Pink ppt. (dissolved and refractionated in same manner)
1300 ml. clear red supernatant
Add 1 N HCl dropwise with stirring to pH 4.20 as measured by glass electrode
Clear yellow supernatant (discarded)
Red ppt. containing 100 to 120 mg. microsomal cytochrome
(Dissolved in 30 to 50 ml. 0.1 M Tris, pH 7.5)

DIAGRAM 1. Step 5. Ammonium sulfate fractionation. All centrifugations are at 11,000 × *g* for 10 minutes. Abbreviations: SAS, saturated ammonium sulfate; AS, solid ammonium sulfate.

70 mg., was used in the experiments described in this paper. It could be stored at 5° in 0.1 M Tris buffer, pH 7.5, for at least 2 weeks without change in spectra or enzymatic properties, or kept frozen at −20° for longer periods of time.

Properties of Microsomal Cytochrome

Absorption Spectra—Fig. 1 shows the absorption spectra of a typical preparation of microsomal cytochrome in the oxidized and reduced forms. The reduced spectrum in the visible region is the same with a wide variety of reducing agents (see "Oxidizing and reducing agents"). In the example illustrated, excess cysteine was selected as reducing agent because it causes no appreciable spectral interference at wave-lengths higher than 300 mμ. The millimolar absorption coefficients, E_{mM}, were based upon heme content as described in the section on the calculation of E_{mM} values. In the Soret region, a 10 mμ shift occurs on reduction similar to that obtained in suspen-

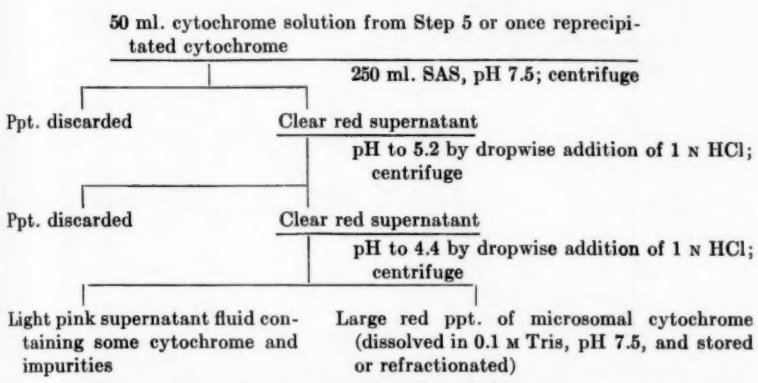
50 ml. cytochrome solution from Step 5 or once reprecipitated cytochrome
250 ml. SAS, pH 7.5; centrifuge
Ppt. discarded
Clear red supernatant
pH to 5.2 by dropwise addition of 1 N HCl; centrifuge
Ppt. discarded
Clear red supernatant
pH to 4.4 by dropwise addition of 1 N HCl; centrifuge
Light pink supernatant fluid containing some cytochrome and impurities
Large red ppt. of microsomal cytochrome (dissolved in 0.1 M Tris, pH 7.5, and stored or refractionated)

DIAGRAM 2. Refractionation of microsomal cytochrome. The pH measurements were made with dipping electrodes. All centrifugations were at 11,000 × *g* for 10 minutes. Abbreviations: SAS, saturated ammonium sulfate; AS, solid ammonium sulfate.

sions of microsomes (2). The E_{mM} value at the Soret maximum increases from 117 in the oxidized form to 171 in the reduced form. From 300 to 600 mμ, the spectra correspond closely to those reported by Appleby and Morton (10) for the flavin- and heme-containing lactic dehydrogenase of yeast, although, as will be shown, the microsomal cytochrome contains no flavin. The absorption peaks at 526 and 556 mμ are in agreement with the maxima reported for microsomal suspensions (3). In addition, extension of spectral observations to the 300 to 400 mμ region shows broad absorption bands for the oxidized and reduced forms at 355 to 370 mμ and from 320 to 340 mμ, respectively. These bands, although broad, are comparable with the 526 and 556 mμ peaks in intensity.

Identity of Porphyrin—The heme group of the cytochrome is readily and quantitatively removed from the protein by an acid acetone method similar

to that of U. J. Lewis (11). The coincidence of the absorption spectrum of the heme with that of ferriprotoporphyrin chloride, Fig. 2, suggests the identity of the two compounds and prompted us to use the extinction coefficients reported by U. J. Lewis to make a quantitative determination of the isolated heme spectrophotometrically.

Analysis for Iron and Heme and Calculation of E_{mM} Values—Various cytochrome preparations were dialyzed against 100 to 200 volumes of 0.001 M Tris buffer, 0.001 M cysteine, pH 7.0, or glass-distilled water for 36 hours with three changes of water or buffer. The heme protein bonds were bro-

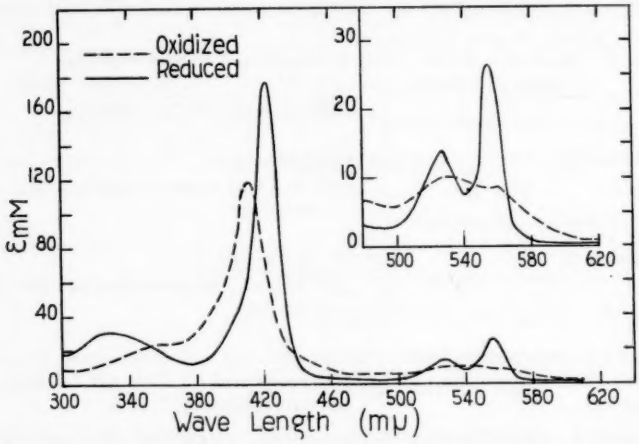

FIG. 1. Absorption spectra of oxidized and reduced microsomal cytochrome. Optical density readings were taken at 5 mμ intervals over the entire wave-length range and at 1 mμ intervals at each absorption maximum or minimum. The protein spectra were read in 0.10 M Tris, pH 7.35; for reduction 9 μmoles of cysteine per ml. were added. The control tube contained the same buffer and the same amount of cysteine. E_{mM} values were based on heme analyses by the method of Lewis (11).

ken at or below pH 1 with hydrochloric acid at room temperature, and the protein was precipitated with approximately 20 volumes of redistilled acetone. After removal of the colorless protein by centrifugation, the supernatant fluid and acetone washings were diluted to volume with acetone for spectrophotometry. As a check on the heme analyses, analyses on the acid-acetone method were run on recrystallized horse oxyhemoglobin. These analyses agreed within 5 per cent with the calculated value based upon the oxyhemoglobin absorption bands. Alkaline pyridine and cyanide hemochromogen derivatives, prepared in the usual way without prior separation of the protein, gave bands in the proper position, but were not wholly satisfactory. The apparent molar absorption coefficients as

well as details of the spectra are influenced by the protein as shown by variations reported for different heme proteins carrying the same heme group (12).

Iron analyses, with reduced iron and also Mohr's salt for preparing standard solutions, were carried out on the dialyzed heme protein by the method of Drabkin (13). The same method, involving wet ashing and orthophenanthroline for color development, was employed on the quantitatively isolated heme after evaporation of the acetone. The results by both procedures were the same. A commercial preparation of cytochrome *c*, 0.34

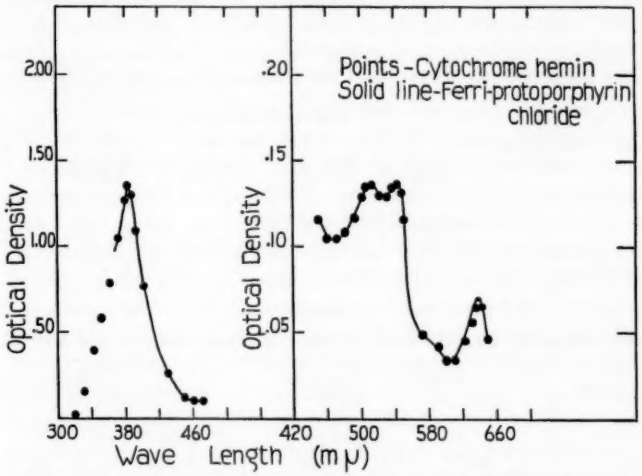

FIG. 2. Comparison of absorption spectra of acetone-HCl derivative of microsomal cytochrome and ferriprotoporphyrin. The ferriprotoporphyrin chloride spectrum according to Lewis (11).

per cent iron, gave concordant values by wet ashing and by ignition. The spectra of all solutions subjected to analysis were measured.

The millimolar absorption coefficients based upon iron content and upon heme are presented in Table I. The agreement between the values of E_{mM} based upon heme and upon iron analyses is adequate. Variations of ±8 per cent in the values based on iron and ±4 per cent in the values based on heme may be, in part, of analytical origin. However, the different preparations may not be completely uniform. It is difficult to exclude small and variable traces of non-heme iron, and it is possible that a small amount of denaturation occurs during the acid precipitation step.

Dry Weight and Minimal Molecular Weight—In Table II are the results of dry weight determinations and the minimal molecular weights calculated

upon the basis of the dry weights and the E_{mM} values of the reduced cytochrome at 423 mμ. The protein samples, after prolonged dialysis against glass-distilled water, were dried *in vacuo* at room temperature and then at 110° to constant weight. Although these calculations are subject to the cumulative errors in E_{mM}, in drying and weighing small samples, and in possible contamination by salts and residual water, the results are in agreement with those obtained from sedimentation and diffusion analyses as described in the section on molecular weight.

Sedimentation Coefficient—Two solutions of the cytochrome, 0.2 per cent protein in 0.1 M acetate, pH 6.95, and 0.4 per cent protein in 0.1

TABLE I

Absorption Coefficients for Microsomal Cytochrome Based on Iron and Heme Analysis

Analysis	No. of preparations analyzed	E_{mM} of absorption peaks				Iron / Heme
		Oxidized, 413 mμ	Reduced, 423 mμ	Reduced, 526 mμ	Reduced, 556 mμ	
Iron	8	113 ± 10	165 ± 15			
Heme	4	117 ± 5	171 ± 8	13.4 ± 0.5	25.6 ± 1.0	1.04

TABLE II

Minimal Molecular Weight Based on Iron and Heme Analyses and Dry Weight

Preparation No.	Optical density at 423 mμ	Cytochrome concentration*	Dry weight	Minimal molecular weight
		μmole per ml.	*mg. per ml.*	
19	10.3	0.058	0.942	16,200
44	31.6	0.185	3.55	19,200

* Based on E_{mM} = 171 at 423 mμ for the reduced form.

ionic strength phosphate, pH 7.38, sedimented with a single boundary in prolonged sedimentation runs (5 to 6 hours) at 5° in the analytical ultracentrifuge. The results in phosphate, corrected to an average rotor temperature of 10° in water, give a sedimentation coefficient $s_{10,w}$, of 1.0 × 10^{-13} sec. $^{-1}$.

Diffusion Coefficient—The diffusion coefficient was determined with a 0.3 per cent protein solution in 0.1 ionic strength phosphate, pH 7.38, in duplicate. Boundaries were formed in both limbs of a standard Tiselius electrophoresis cell (Klett) and, after compensation, were followed at intervals with Longsworth schlieren scanning photographs at 2°. Normalized curves at four time intervals fell within close limits on the Gaussian distribution curve. The diffusion coefficients, $D_{10,w}$, calculated by the maximal

ordinate and the maximal ordinate-area methods (14), by using sixteen time points, are 5.57×10^{-7} and 5.48×10^{-7} cm.^{2} sec.$^{-1}$, respectively. For comparison with sedimentation coefficients these measurements, made near 2°, were extrapolated to the conditions prevailing in water at 10°.

Partial Specific Volume—This quantity ($\bar{V}$) was determined by the density gradient method (15) and found to be 0.748.

Molecular Weight—By using the above quantities in the standard relation for molecular weight (14), a value of 16,900 is obtained for the molecular weight. From this value and the partial specific volume, one may calculate the radius of the sphere of equivalent volume, and correspondingly the diffusion coefficient, D_0, for the equivalent sphere in water. The ratio between this value and the observed coefficient is 1.7, indicating a high degree of molecular asymmetry.

Electrophoretic Analysis—Experiments were carried out in the pH range 6.9 to 7.5 with 0.1 M Tris, 0.1 ionic strength phosphate, and 0.1 M acetate buffers. The single ascending boundary in these experiments remained sharp and symmetrical for 150 to 300 minutes. The descending boundary broadened more rapidly and, after 3 to 4 hours, showed indication of two very slowly separating heme protein components in an area ratio of roughly 3:2. In view of the spectral evidence presented here and in following papers (5, 16), we consider the inhomogeneity to represent minor charge modifications of a single heme protein species. Although no evidence of a more fundamental inhomogeneity has been detected, the problem awaits more detailed investigation.

Over the limited pH range studied, the protein carried a relatively high, net negative charge. The mobility at pH 7.5, in 0.1 M Tris chloride buffer, is -5.9×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$. In view of the mobilities and of the fact that the pH of water-dialyzed solutions becomes stabilized between pH 5 and 6, it is likely that the isoelectric and isoionic regions are below pH 6. It should be noted in this respect that the solubility of the protein in concentrated ammonium sulfate solutions is greatly diminished at pH 5 and below, a property utilized in the purification.

Flavin Analysis—Flavin analyses were carried out by the sensitive fluorometric method of Burch, Bessey, and Lowry (17). The results, for which we are indebted to Dr. Helen B. Burch, reveal no more than 0.5×10^{-4} molecule of flavin per molecule of cytochrome. It is concluded that flavin does not form an integral part of this protein. This is in contrast to the yeast lactic dehydrogenase (10) which exhibits a similar spectrum but contains flavin and heme in a 1:1 ratio.

Stability—At neutral and alkaline pH the cytochrome is stable. The spectrum remains unchanged for at least several hours at 20–37°, pH 7.5. Preparations have been incubated for 1 hour at 25° at pH 8.1 to 9.5 with

no appreciable change in the oxidized or reduced spectra. Neutral solutions can be stored for at least 2 weeks at 5° and much longer at −20°. The protein is less stable, however, in the acid pH region. When samples of the cytochrome were incubated at 25° for 10 minutes at pH 6.5, 4.3, and 3.5, the amounts of denaturation as measured by loss of cytochrome spectrum were 0, 30, and 100 per cent, respectively. This denaturation is very much depressed at low temperatures (5°) and at high salt concentration (90 per cent ammonium sulfate saturation), and it is for this reason that the acid precipitation of cytochrome in the isolation procedure entails little loss of material.

Effects of Various Reagents—*N*-Ethyl maleimide, 10^{-3} M, incubated with the protein at pH 7.4 for 24 hours at 5°, and *p*-chloromercuribenzoate, 10^{-3} M, had no effect on the oxidized or reduced spectra in the 400 to 600 mμ range, indicating that no thiol groups susceptible to these reagents affect the heme. Hydroxylamine, 0.1 M, pH 7.4, and hydrogen peroxide, 0.1 M, pH 8.0, were similarly without effect. Hydrogen peroxide, however, oxidized the reduced form. No significant effect on the oxidized or reduced spectra were obtained with 0.1 M sodium cyanide, pH 8.0, or in solutions kept in an atomosphere of carbon monoxide at pH 8.0 for 3 days at 5° or for 1 hour at 25°. Except where otherwise indicated, these reagents were incubated with the cytochrome for 15 to 30 minutes at 25° under anaerobic conditions. In view of the slight spectral effects observed by Horecker and Kornberg (18) with cytochrome *c* and sodium cyanide, a more detailed examination of this reagent with the microsomal cytochrome should be undertaken. It is especially noteworthy that carbon monoxide does not alter the absorption spectrum of either the oxidized or reduced form even on prolonged equilibration.

Oxidizing and Reducing Agents—The microsomal cytochrome at pH 7 to 8 is reduced by reduced indigo di-, tri-, and tetrasulfonates, anthraquinone-2,7-disulfonate, and benzyl viologen, and by sodium hydrosulfite and cysteine. Potassium borohydride at pH 5.5 is also an effective reducing agent. In all cases the spectra obtained were identical, in those regions where comparison was possible, with that shown in Fig. 1. Large excesses of reduced glutathione caused no appreciable reduction. Neither DPNH nor TPNH alone reduces the oxidized cytochrome. Rapid and complete reduction of the cytochrome by these coenzymes is catalyzed by reductases that have been obtained in soluble form from microsomes. The spectrum of the cytochrome reduced enzymatically is the same as that observed with the various other reducing agents. The liberation of the TPNH enzyme and the preparation and properties of the DPNH-specific reductase are described in a following report (16).

Reduced microsomal cytochrome is oxidized rapidly by potassium fer-

ricyanide, ferric chloride, cytochrome *c*, and various dyes of appropriate potential. It is oxidized somewhat more slowly by oxygen and by mercuric chloride to give the oxidized spectrum shown in Fig. 1.

DISCUSSION

The liberation of the microsomal cytochrome from its particulate complex appears to be primarily lipolytic, although the possibility of some proteolysis has not been rigorously excluded. In so far as comparison with the particulate pigment is possible (2–4), no significant alteration of properties has been detected with the exception of the apparent standard potential. This potential determination and its interpretation are treated in the following paper (5). The isolated protein, by spectral criteria, appears homogeneous with respect to its heme properties. Since the material sediments as a single compound, the apparent electrophoretic inhomogeneity may result from minor charge modification of the same substance, similar perhaps to the heterogeneity of crystalline insulin which may vary in its content of amide nitrogen (19). The agreement between molecular weight and the minimal molecular weight based on iron and heme analysis indicates the presence of one heme per molecule and no significant amount of nonheme iron, and provides some stoichiometric evidence for homogeneity. Particular attention was paid to establishing molar absorption coefficients, since these provided the experimental basis for work to be described and since cytochrome *c* is the only other mammalian cytochrome for which such properties had been directly determined.

The spectra of the cytochrome, in both its oxidized and reduced forms, resemble those of other heme proteins which, on the basis of magnetic susceptibility measurements, have been considered to coordinate iron by bonds of predominantly covalent character. The inertness to a variety of reagents that attack heme iron is consistent with this classification. Failure to react with carbon monoxide during prolonged exposure is of particular significance in this respect. The protein is quite stable in the alkaline pH region and shows no spectral changes as a function of pH up to pH 10. Spectral changes in the acid pH region are associated with the breaking of the heme protein bonds, as indicated by the complete resolution of heme and protein by the acid acetone method. This behavior is in contrast to that of cytochrome *c*, in which the heme is bound to the protein by thio ether linkages and undergoes fully reversible changes down to pH 1.

SUMMARY

1. The cytochrome of rabbit liver microsomes has been isolated by treatment with pancreatic lipase and purified by ammonium sulfate fractionation at controlled pH.

2. The purified protein sediments in the ultracentrifuge as a single protein. From sedimentation velocity, diffusion, and partial specific volume measurements, the molecular weight is about 17,000.

3. Quantitative analyses for iron and heme indicate the presence of one heme per molecule and no non-heme iron.

4. The spectrum of the isolated ferriporphyrin chloride in acetone is identical with that of the ferriprotoporphyrin chloride obtained from hemoglobin. Unlike cytochrome *c*, the heme and protein of the microsomal cytochrome are readily separated by acid acetone treatment.

5. The spectrum of the purified microsomal cytochrome, intermediate between that of cytochrome *c* and particulate mitochondrial cytochrome *b*, gives no evidence of more than one heme protein component, a finding supported by the stoichiometry and the sedimentation analysis.

6. Prolonged electrophoresis reveals the possible presence of two heme protein components distinguishable only by a very small difference in net charge.

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THE OXIDATION-REDUCTION STOICHIOMETRY AND POTENTIAL OF MICROSOMAL CYTOCHROME*

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In the preceding report (1) we have described the isolation, as an acidic protein of relatively low molecular weight, of the cytochrome component of rabbit liver microsomes. Of the various mammalian cytochromes, known from their spectra in crude preparations or intact cells, only cytochrome *c* has been previously isolated in a form which has permitted molecular characterization (2–4). Two properties of cytochrome *c*, its standard potential and its behavior as a univalent electron acceptor (5), have been major considerations in theories of metabolic electron transport. This paper deals with the potential and the stoichiometry of the oxidation and reduction of the microsomal cytochrome.

Methods

Potentials of the cytochrome were calculated from equilibrium measurements with oxidation-reduction couples of known standard potential. The cytochrome was prepared and analyzed by methods which have been described (1). Of the oxidation-reduction dyes in suitable potential range for equilibrium measurements, indigo tetrasulfonate, which has been well characterized potentiometrically (6), proved most satisfactory. The indigo sulfonates used were samples prepared and analyzed by Dr. P. W. Preisler. The second equilibrium system used was that established between the cytochrome and mixtures of ferrous and ferric oxalate. Potentials of the ferrous-ferric oxalate couple were reported by Michaelis and Friedheim (7). Ferrous and ferric oxalates have been used in cytochrome equilibria by Hill (8).

Anaerobic Cuvettes—Since many of the reaction systems studied were autoxidizable in air, anaerobic conditions had to be maintained. Two types of reaction vessel were employed. The first consisted of a square Corex cuvette of 1 cm. light path fused to a Thunberg bulb and side arm. The second type of cell was a 3 ml. Corex or silica cuvette with a ground glass joint and upper assembly as illustrated in Fig. 1. This type of cell was used in anaerobic titrations. Additions were made with a calibrated

* This work was aided by a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council.

micro syringe and a No. 22 needle inserted and left in position in the rubber injection port. Increments of 0.02 ml. had a precision of about ±5 per cent, but, since straight lines could be fitted to a sequence of five to fifteen points, the over-all precision was better than 5 per cent.

In practice a small volume of cytochrome solution was pipetted into the cell. The upper assembly was inserted and the system was alternately evacuated on the water pump and flushed with nitrogen. Buffer, deaerated

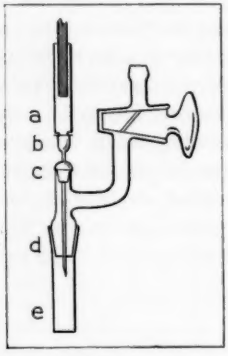

FIG. 1

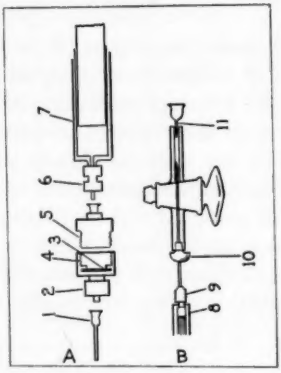

FIG. 2

FIG. 1. Cuvette and upper assembly used in spectrophotometric titrations under anaerobic conditions. *a*, 1 ml. syringe graduated in 0.01 ml. units; *b*, No. 22 stainless steel needle; *c*, rubber injection port; *d*, 12/10 standard taper joint; *e*, 1 cm. square cuvette, 3 ml. capacity.

FIG. 2. *A*, syringe filter assembly. *1*, No. 22 needle; *2* and *6*, Luer-Lok needle sockets; *3*, asbestos filter on stainless steel gauze; *4*, gasket; *5*, screw seal; *7*, 5 or 10 ml. syringe. Swinney filter adapter obtained from Becton, Dickinson, and Company. *B*, transferring coupler. This is used in transferring leuco dye anaerobically from storage syringe, *7*, to 1 ml. titration syringe, *8*. The No. 22 needle, *9*, of the 1 ml. syringe passes through the rubber injection port, *10*, and through the 1 mm. bore capillary and stop-cock. Cut-off needle, *11*, is cemented into the capillary.

with a vigorous stream of nitrogen, was added from a syringe through the injection port. The system was again alternately evacuated and flushed several times. Nitrogen was left in the gas space during an experiment. Even though quite small volumes of reagent were added in oxidative titrations, the solutions employed had to be deoxygenated. Mixing, after addition of reductant or oxidant, was effected by inversion of the cuvette assembly. With Linde nitrogen and the above precautions, the titration blanks were negligible, and the reduced cytochrome was stable.

Leuco Dyes—Dye solutions of somewhat higher than the desired final concentrations were reduced with hydrogen and a supported palladium

catalyst. These reductions were carried out with the solutions in the cylinder of a 5 or 10 ml. syringe through which a stream of hydrogen gas was bubbled. After reduction, the plunger was rapidly inserted and the hydrogen bubble and some of the solution were ejected through the filter assembly illustrated in Fig. 2. When not in use the needle was sealed by insertion in a soft rubber stopper. Concentrations of the total and reduced dye were determined spectrophotometrically before and after reoxidation. Final concentrations were adjusted by sucking the appropriate amount of deoxygenated buffer into the syringe. In equilibrium titrations an excess of the reduced dye was injected directly from the storage syringe into the anaerobic cuvette containing the cytochrome solution. A series of equilibria was then established by addition of increments of standard ferricyanide.

TABLE I

Millimolar Absorption Coefficients Used in Equilibrium Calculations

Substance	pH	E_{mM}		
		590 mμ	423 mμ	413 mμ
Indigo tetrasulfonate, oxidized	5.5	22	0.5	0.6
" " "	6.3	22	0.5	0.6
" " "	6.9	22	0.5	0.6
" " reduced	5.5	0	7.5	12.5
" " "	6.3	0	8.9	13.8
" " "	6.9	0	12.5	14.7
Microsomal cytochrome, oxidized		0	65	117
" " reduced		0	170	73

When the leuco dye was used as a reductive titrating agent it was first transferred to a smaller syringe through the coupler shown in Fig. 2, *B*. Other solutions used in anaerobic work were deoxygenated and stored in small stoppered separatory funnels fitted at the tips with rubber injection ports for the removal of samples with a syringe and needle.

Absorption Coefficients—Absorption coefficients, determined on dye solutions standardized by weight, were checked by stoichiometric titration of the leuco dye in anaerobic cuvettes with standard ferricyanide. In a 2 electron change the titration slope, $\Delta \log(I_0/I)$ μeq.$^{-1}$ ml.$^{-1}$, is one-half the difference between the millimolar absorption coefficients of the oxidized and reduced forms of the dye at the wave-length investigated. The absorption coefficients of dye and cytochrome used in the equilibrium measurements are given in Table I. From optical density measurements at three wavelengths, it was possible to calculate the concentrations of three of the four reacting components in the cytochrome-dye equilibrium. The correct ab-

solute values of the absorption coefficients are unimportant in the equilibrium measurements, provided that the ratios between them are accurately known.

EXPERIMENTAL

Oxidative and Reductive Titrations—Buffered cytochrome solutions in anaerobic cuvettes were reduced by addition of increments of deoxygenated solutions of sodium hydrosulfite, leucoanthraquinone-2,7-disulfonate, or leucoindigo disulfonate. At pH 5.5, reduction could be effected by injection of 0.02 ml. of potassium borohydride, 20 mg. per ml. at pH 9, into the deoxygenated cytochrome solution. At the acid pH, the borohydride rapidly decomposes, reducing the cytochrome and liberating the hydrogen. After reductions with borohydride, the cuvette was repeatedly evacuated and flushed with nitrogen until no more bubbles were formed. Reductions were also carried out enzymatically with reduced diphosphopyridine nucleotide and the microsomal enzyme described in the following report (9). Cytochrome reduced by these various methods between pH 5 and 8 was titrated with standard ferricyanide. In those cases in which an excess of reducing agent was employed, there was an initial plateau in which increments of ferricyanide had no effect upon the spectrum. When excess reducing agent was consumed or if none was initially present, the optical densities at wave-lengths affected by the state of oxidation changed linearly with increments of ferricyanide until the cytochrome was completely reoxidized. The results of a titration after hydrosulfite reduction are shown in Fig. 3. In this titration the optical density of the solution was measured at 413, 423, 527, and 555 mμ after each increment of ferricyanide. Within the limits of experimental error the curves, after correction for volume changes, are linear, show no significant breaks, and begin and end abruptly. The linearity of the titration and the fact that the absorption bands move in unison indicate the presence of a single heme protein. Moreover, no evidence has been obtained in such titrations for the existence of any intermediate spectra that cannot be accounted for as the simple sum of the contributions of the fully oxidized and fully reduced forms present.

Similar titrations have been carried out by starting with the cytochrome in the oxidized form and by using hydrosulfite or leucoanthraquinone-2,7-disulfonate as the titrating agent. Freshly prepared deoxygenated solutions of these substances were standardized immediately before and after use by spectrophotometric titration of indigo tetrasulfonate. Occasional reductive titrations of the cytochrome exhibited a variable initial plateau owing to the presence of traces of oxygen or other reducible impurities. No consistent evidence could be obtained by this method for the existence of any reducible group on the protein other than the prosthetic group. The

reductive titration curves were linear with the same slope exhibited in the oxidative titrations. Several consecutive cycles of reduction and oxidation were carried out with the same or different reagents with no significant change in the slopes.

The slope of an oxidative or reductive titration curve, $\Delta \log (I_0/I)$ μeq.$^{-1}$ ml.$^{-1}$, for a cytochrome exhibiting a 1 electron change, should be numerically equal to the difference between the millimolar absorption coefficients

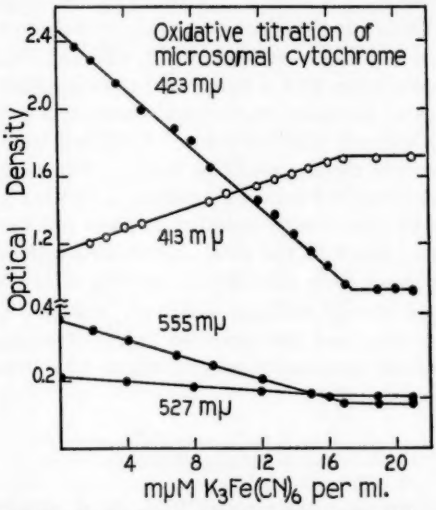

FIG. 3. Oxidative titration of microsomal cytochrome with potassium ferricyanide after reductive titration with sodium hydrosulfite. The ordinate, D_λ, is the optical density at the wave-length, $\lambda(m\mu)$, which is indicated numerically at each curve. The solution contained 0.05 M potassium phosphate buffer, pH 7.0. The initial volume was 2.25 ml., and reduction was effected by stepwise addition of 0.03 ml. of sodium hydrosulfite solution under anaerobic conditions. Reoxidation was effected with 2×10^{-4} M potassium ferricyanide. All optical density readings are corrected for dilution by the oxidant.

of the oxidized and reduced forms. In twenty-five oxidative titrations in the pH range 5 to 8 on six different cytochrome preparations, the average slope at 423 mμ was 91 with a standard deviation of 7. The slopes of a smaller number of reductive titrations fell within these limits. The difference in millimolar absorption coefficients at this wave-length is 105. To what extent the deviation between these figures results from inaccuracies in the methods or from interfering groups or impurities is not yet known. The oxidation and reduction correspond to the removal and addition of 1 electron, determined with an over-all accuracy of ± 10 per cent. Oxidative

titration of reduced cytochrome *c*, followed at 550 mμ, showed slopes of 18.4 to 18.6, whereas the difference in millimolar absorption coefficients of the reduced and oxidized forms at this wave-length has been reported to be about 18.9 (10).

Equilibrium with Indigo Tetrasulfonate—Indigo disulfonate had too negative a potential to establish easily measurable equilibria with the free cytochrome, but indigo tetrasulfonate, E_0' −0.046 at pH 7, established measurable equilibria between pH 5 and 7. A 10- to 20-fold excess of the reduced dye was injected into a buffered solution of the cytochrome in an anaerobic cuvette. Optical densities were read at 413, 423, and 590 mμ. A syringe containing approximately 10^{-4} M ferricyanide was inserted in the injection port and readings at the above wave-lengths were taken after each successive increment of 0.02 ml. of ferricyanide. Equilibria were established and stable before the first reading could be taken. The concentration of the oxidized dye was computed from the reading at 590 mμ. The concentration of the reduced dye at each equilibrium point was obtained by difference between this value and the total dye concentration checked at the end of the experiment from the 590 mμ reading after complete reoxidation. The optical density readings at 413 and 423 mμ were corrected for the dye contributions, and the corrected values were used to compute reduced and oxidized cytochrome concentrations by solution of the simultaneous equations

$$D_{413} = C_o E_{413,o} + C_r E_{413,r} \quad (1)$$

$$D_{423} = C_o E_{423,o} + C_r E_{423,r} \quad (2)$$

where D is the corrected optical density at the designated wave-length, E is the millimolar absorption coefficient, C is the concentration of cytochrome, and the subscripts o and r refer to oxidized and reduced forms. As a check on the measurements and calculations, the sums of the concentrations of the two forms of cytochrome must agree with the initial concentration and the final concentration rechecked at 413 mμ after complete reoxidation.

The results of the equilibrium titration at pH 5.5 are shown in Table II. K', the equilibrium constant, is seen to hold within reasonably close limits between 25 and 83 per cent oxidation of the cytochrome when the reaction is formulated as

$$(\text{Dye})_r + 2(\text{cytochrome})_o = (\text{dye})_o + 2(\text{cytochrome})_r \quad (3)$$

$$K' = \frac{[\text{dye}]_o}{[\text{dye}]_r} \times \left(\frac{[\text{cytochrome}]_r}{[\text{cytochrome}]_o}\right)^2 \quad (4)$$

Thus the equilibria are in accord with the titration stoichiometry and indicate a 1 electron change in the cytochrome.

The equilibria may also be expressed in terms of potentials. In this method the dye is treated as an oxidation-reduction indicator. At each equilibrium point the potential of the indigo tetrasulfonate referred to the

TABLE II

Oxidation-Reduction Equilibria between Microsomal Cytochrome and Indigo Tetrasulfonate at pH 5.5

Equilibrium No.	$[Dye]_o$	$[Dye]_r$	$[Cytochrome]_o$	$[Cytochrome]_r$	$[Cytochrome]_o + [Cytochrome]_r$	K'
	μM*	μM	μM	μM	μM	
1	7.5	58.7	2.67	6.72	9.39	0.58
2	10.5	55.4	3.22	6.30	9.52	0.73
3	13.4	52.8	3.84	5.66	9.50	0.55
4	16.3	49.9	3.96	5.52	9.48	0.63
5	19.4	46.8	4.22	5.14	9.36	0.59
6	22.7	43.5	4.57	4.83	9.40	0.58
7	26.2	40.0	4.70	4.63	9.33	0.63
8	30.0	36.2	5.07	4.32	9.39	0.60
9	33.9	32.3	5.56	3.92	9.48	0.52
10	38.1	28.1	5.75	3.63	9.38	0.54
11	41.6	24.6	6.12	3.58	9.70	0.58
12	45.4	20.8	6.40	3.13	9.53	0.52
13	49.1	17.1	6.66	2.85	9.51	0.52
14	53.2	13.0	7.02	2.53	9.55	0.53
15	56.6	9.6	7.30	2.04	9.34	0.46

The titration was carried out under anaerobic conditions at 26°. The solution, 2.00 ml. initial volume, contained 0.1 M sodium Versenate, pH 5.5, 9.45 mMmoles of microsomal cytochrome per ml., and 66.2 mμmoles of indigo tetrasulfonate, oxidized plus reduced, per ml. Successive equilibria were established by injection of increments of 0.7×10^{-3} M potassium ferricyanide, 0.02 ml. in most cases.

* Observed optical densities were corrected for dilution by the oxidant. The recorded concentrations are thus corrected to correspond to the original 2.00 ml. volume. This does not affect the calculation of K' and facilitates the summation in the sixth column as a check on the analysis and the recovery of the cytochrome.

normal hydrogen electrode is given by the relation

$$E = E_0' + \frac{RT}{2F} \ln \left(\frac{[dye]_o}{[dye]_r} \right) \quad (5)$$

and can be computed since E_0' as a function of pH is known (6) and the concentration term is measured. At equilibrium, the cytochrome potential is the same as that of the dye. Accordingly in Fig. 4, the computed value of E at each equilibrium point is plotted against the fraction of the cytochrome in the oxidized form. The solid curves are the theoretical curves for a substance undergoing a 1 electron change, corresponding to a numeri-

cal factor of 1 instead of 2 in the denominator of the second term on the right in Equation 5. As would be expected from the data in Table II, the points fall on the potential curves for a 1 electron change. E_0' is the potential at the mid-point of the curve. As the pH is increased, the E_0' of the cytochrome and that of the dye diverge so that the range of the titration curve that can be covered by equilibrium measurements becomes progressively more limited. At pH 6.9 it is necessary to extrapolate to the

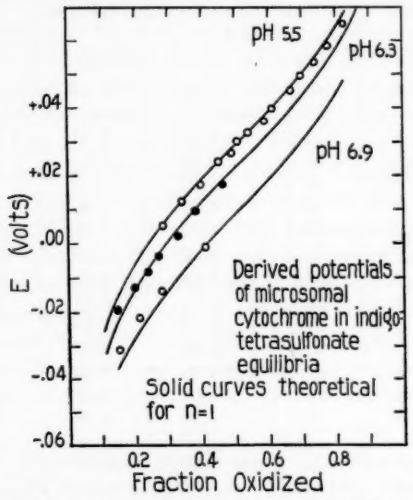

FIG. 4. Potentials of the microsomal cytochrome calculated from equilibria with reduced and oxidized indigo tetrasulfonate at 26°. Temperature in these and other experiments was controlled with thermal spacers around the carriage compartment of the spectrophotometer. These were connected to a circulating water bath.

mid-point from experimental points which do not quite fit the theoretical curve.

Equilibria with Ferrous and with Ferric Oxalate—The E_0' of the ferrous-ferric oxalate couple is close to zero below pH 7 and is independent of pH. Near pH 7, the E_0' curve assumes a slope of -0.06 volt per pH unit according to potentiometric measurements (7). Using the equilibrium titration method described in the preceding section, we measured equilibria between ferrous and ferric oxalate and the indigo tetrasulfonate couple over the pH range 5.2 to 8.5. The values of E_0' for the ferrous-ferric oxalate couple calculated from these measurements were in fair agreement with the potentiometric values. Corresponding experiments were therefore undertaken with the cytochrome in place of the dye.

Buffered solutions of the cytochrome containing 0.5 M sodium oxalate and 0.001 M ferric ammonium sulfate were prepared in anaerobic cuvettes. Measured increments of freshly prepared deoxygenated ferrous ammonium sulfate were then added from a syringe, and readings were taken after each addition at 413 and 423 mμ. The potential at each equilibrium point was calculated from the E_0' and known concentrations of ferric and of ferrous oxalate by the relation analogous to Equation 5 for a 1 electron change. Reduced and oxidized cytochrome concentrations were computed as before by Equations 1 and 2. The E_0' values of the cytochrome derived from

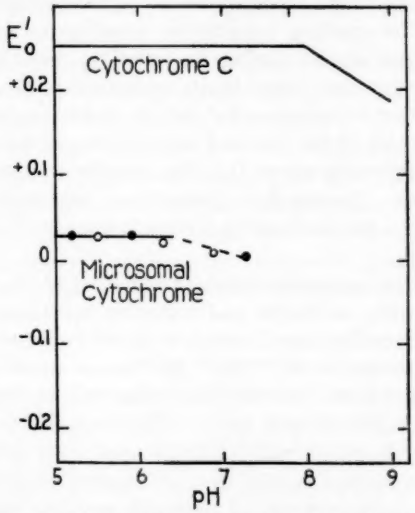

FIG. 5. The apparent standard potential, E_0', of the microsomal cytochrome as a function of pH at 26°. ○, indigo tetrasulfonate equilibria; ●, ferric-ferrous oxalate equilibria. The upper curve is from the data of Rodkey and Ball (5).

these results and from the indigo tetrasulfonate equilibria are shown in Fig. 5 as a function of pH. Between pH 5.2 and 6.3 the results by the two methods are in fair agreement and indicate a potential, E_0', between +0.03 and +0.025 volt for the free cytochrome. Since the criteria for equilibria were satisfactory and since the concentrations of all of the reacting components were measured, the assignment of the cytochrome potential depends chiefly upon the published standard potentials of the reference compounds. Internal consistency between the published values for ferrous-ferric oxalate and indigo tetrasulfonate was established by titrating them against each other. At pH values above 6.5 there is indication of a negative slope in the E_0' *versus* pH curve for the cytochrome which is

smaller than that predicted for either a 1 or a 2 electron change. Because of limitations of the methods in this pH region, we are reluctant to accept or interpret any apparent E_0' *versus* pH slope for the cytochrome at the present time. Spectral criteria and stoichiometric titrations are in accord with a 1 electron change for the cytochrome, both in the acid and in the alkaline pH regions.

DISCUSSION

The potential of the free microsomal cytochrome at pH 7 is more than 0.1 volt higher than values reported for the pigment in particulate suspension (11, 12). The question is naturally raised as to whether the cytochrome has become altered during its isolation, possibly in the incompletely defined enzymatic step in its preparation (1). It should be noted, however, that no evidence for such a change is provided by comparison of the spectra of the free and bound forms of the cytochrome (1). As shown in the following report (9), the cytochrome also has specific enzymatic properties. Aspects of similar problems concerned with the status of free cytochrome *c* are discussed in a recent paper by Keilin and Hartree (13).

A difference in the apparent standard potential of the free and bound forms of a reversibly oxidizable and reducible substance is not without precedent. An immediate case in point is the ferric-ferrous oxalate system employed in the present work. The ferric-ferrous couple in oxalate complex has a potential about 0.8 volt lower than values obtained in the absence of such a complex-forming agent. The large shift in the equilibrium of the alcohol dehydrogenase reaction in the presence of stoichiometric concentrations of the enzyme results from differential complex formation of the reduced and oxidized forms of diphosphopyridine nucleotide by the protein (14, 15). A shift would occur in the presence of any differential complex-forming agent, not necessarily the enzyme catalyzing the reaction. In a thermodynamic treatment, the equilibrium shift emerges as a change in standard potential of the nucleotide. The potential shift which occurs on liberation of the microsomal cytochrome from its particulate complex may perhaps be attributed to a differential interaction of the reduced and oxidized cytochrome with one or more components of the microsomal particle. This does not eliminate the problem of possible alteration of the protein during its isolation but raises the question of the nature of the binding. The potential shift may be of purely thermodynamic interest, or it may reflect fundamental differences in the mechanisms of reaction of the free and bound cytochrome.

The free microsomal cytochrome, like cytochrome *c*, behaves as a univalent electron donor and acceptor. The idea that electrons move, one at

a time, through a cytochrome sequence, extrapolated from the behavior of cytochrome *c*, has posed one of the many difficult problems in the understanding of the chemical coupling between electron transport and biochemical synthesis, since the latter process is most readily understood in terms of a 2 electron reaction.

SUMMARY

1. Methods are described for carrying out spectrophotometric, oxidative, and reductive titrations of autoxidizable compounds under anaerobic conditions. These methods have been applied to the low molecular weight cytochrome isolated from liver microsomes.

2. The absorption bands of the cytochrome move linearly and in unison with increments of an oxidizing or reducing agent. This is interpreted as evidence for the presence of a single heme protein.

3. Oxidation-reduction equilibria have been measured between the cytochrome and indigo tetrasulfonate and the cytochrome and ferrous-ferric oxalate. From the equilibrium measurements the standard potential, E_0', of the cytochrome at pH 7 is calculated to be +0.02 volt.

4. From stoichiometric, oxidative, and reductive titrations and from oxidation-reduction equilibria the cytochrome behaves as a univalent electron donor and acceptor.

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XUM

A MICROSOMAL CYTOCHROME REDUCTASE SPECIFIC FOR DIPHOSPHOPYRIDINE NUCLEOTIDE*

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Intact liver microsomes catalyze a rapid reduction of cytochrome *c* by DPNH (1, 2).[1] Several workers have studied both this cytochrome *c* reduction and the reduction of the particulate microsomal cytochrome with either DPNH or TPNH as electron donor (3–5). The isolation and characterization of the microsomal cytochrome in free soluble form have been described in the preceding papers (6, 7). This report describes the preparation of soluble protein fractions of microsomes containing all of the reductase activities observed with the intact microsomes, and the isolation and properties of a specific DPNH-microsomal cytochrome reductase. The DPNH-microsomal cytochrome reductase is totally inactive against cytochrome *c*, but, since the reduced microsomal cytochrome reacts rapidly with cytochrome *c*, it completes a DPNH-cytochrome *c* reductase system involving a cytochrome to cytochrome electron transfer.

Methods

The Beckman model DU spectrophotometer, calibrated against several emission bands of a mercury arc, was used for most of the assays. Anaerobic techniques, when required, were similar to those previously described (7). In the case of the microsomal cytochrome, the E_{mM} at 423 mμ during reduction was taken as the average of values obtained from quantitative absorption spectra and titration experiments (6, 7). With assay systems containing both the microsomal cytochrome and cytochrome *c*, the reduction of the cytochrome *c* was followed at 550 mμ. The contribution of the microsomal cytochrome to spectral changes at this wave-length was negligible for the following reasons: (*a*) cytochrome *c* was always present in a 5- to 20-fold molar excess, (*b*) the molar absorption change at 550 mμ is three times greater for cytochrome *c* than for the microsomal cytochrome, and (*c*), during cytochrome *c* reduction with the microsomal cytochrome as

* This work was aided by a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council.

[1] The following abbreviations are used: DPNH, reduced diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; Versene, trade name for ethylenediaminetetraacetic acid; Tris buffer, a buffer prepared from tris(hydroxymethyl)aminomethane; PCMBS, *p*-chloromercuribenzene sulfonate.

XUM

intermediate electron carrier, the microsomal cytochrome is in a predominantly oxidized steady state which changes only slightly throughout most of the reaction (Table I).

The measurement of protein concentration in the purified reductase preparations was based upon the 280 mμ absorption maximum, assuming a specific absorption coefficient of 1 cm.^{2} mg.$^{-1}$. This method was employed only when the preparations were essentially free of nucleotide as judged from the optical density ratios at 260 to 280 mμ.

The various substituted indigo dyes were synthesized by Dr. P. W. Preisler. Other dyes used were commercial preparations from various sources. The beef heart preparation of cytochrome *c* and the PCMBS (8), TPNH, and DPNH were products of the Sigma Chemical Company.

TABLE I

Δ E_{mM} Differences Used in Spectrophotometric Assays

Material reduced	Wave-length	Δ E_{mM} (reduced-oxidized)
	mμ	
Ferricyanide*	340	6.25
Cytochrome *c*	550	18.5
Microsomal cytochrome	423	100
Indigo trisulfonate	600	−21
Indigo tetrasulfonate	590	−22
Thio indigo tetrasulfonate	540	−14.8
Dichlorophenolindophenol	610	−15.5

* Optical density change of DPNH followed.

The ammonium sulfate solutions, sucrose media, and the microsomal cytochrome were prepared as previously described (6).

EXPERIMENTAL

Preliminary Experiments

Initial experiments, in agreement with the results of other workers, showed that microsome suspensions catalyze the rapid reduction of cytochrome *c* by DPNH and TPNH at neutral and slightly alkaline pH values. When the free microsomal cytochrome was tested in such systems, it also was rapidly reduced by both DPNH and TPNH in the presence of a very dilute microsome suspension.

The various ammonium sulfate fractions obtained in the preparation of microsomal cytochrome (6) were examined for activity in the reduction of cytochrome *c* and the microsomal cytochrome. The lipase-treated protein fraction, which precipitated between 50 and 65 per cent saturation of

ammonium sulfate, catalyzed the reduction of both cytochromes with either DPNH or TPNH as electron donor. However, the ratios of activities for DPNH and TPNH and for cytochrome *c* and microsomal cytochrome were found to be variable in several preparations and in various ammonium sulfate fractions of the same preparation.

Further fractionation with ammonium sulfate of the original 50 to 65 per cent saturated ammonium sulfate fraction yielded a preparation which catalyzed cytochrome *c* reduction twenty times more rapidly with TPNH than with DPNH as electron donor. Cytochrome *c* with TPNH as donor was reduced twenty to thirty times more rapidly than the microsomal cytochrome, and added microsomal cytochrome had no effect on the rate of cytochrome *c* reduction. This crude preparation of TPNH-cytochrome *c* reductase may be similar to that isolated by Horecker from acetone powders of whole liver (9). Further discussion of the microsomal-TPNH enzyme will be deferred until further purification and a more critical survey of its properties have been completed.

Whereas the addition of microsomal cytochrome had no effect on the TPNH-cytochrome *c* reductase described above, this was not true of the DPNH activity in the same preparation. The rate of cytochrome *c* reduction was markedly increased by the addition of catalytic amounts of microsomal cytochrome to a system containing DPNH, cytochrome *c*, and catalytic amounts of the 50 to 65 per cent ammonium sulfate fraction. This result suggested that the soluble protein fraction contained an enzyme which was specific for DPNH as donor and the microsomal cytochrome as acceptor. The reduction of cytochrome *c* in such a system could occur by direct electron transfer between the two cytochromes. After examining a number of fractionation methods, the procedure described in the following section yielded preparations of the specific DPNH-microsomal cytochrome reductase.

Preparation of DPNH-Microsomal Cytochrome Reductase from Rabbit Liver

The DPNH-microsomal cytochrome reductase was usually prepared from thirty rabbits. Step 1 is carried out with two rabbits at a time, and the resulting microsome suspensions are stored until all thirty rabbit livers have been carried through this step. Step 3 is very similar to the alcohol extraction procedure used so successfully by Mahler, Sarkar, Vernon, and Alberty (10) in preparing cytochrome *c* reductase from sarcosomes. The temperature is maintained at 3–8° in all the steps unless otherwise indicated.

Step 1—The procedure is identical to Step 1 in the preparation of microsomal cytochrome (6).

Step 2—Approximately 6 to 8 liters of supernatant fluid from Step 1 are carried through this step and Step 3 separately. (This volume choice is limited only by the centrifuges available.) The pH of 8 liters of supernatant fluid is brought to 5.4 by the dropwise addition of 1 N hydrochloric acid, and the mixture is quickly centrifuged to yield a packed precipitate. By using two International centrifuges with six 250 ml. cups at 1500 × *g* for 20 minutes and four Servall centrifuges at 12,000 × *g* for 5 minutes, this is accomplished in approximately 1 hour. The packed pellets are resuspended with a Waring blendor in 3 volumes of distilled water per weight of liver used and again centrifuged in the same way to yield a packed precipitate.

Step 3—The pellets from Step 2, derived from fifteen rabbits, are suspended with a Waring blendor in 500 ml. of 10 per cent ethanol. The temperature is raised to 42–43° in 5 minutes by vigorous stirring in a 65° water bath, maintained at this temperature for 12 minutes with gentle stirring, and finally lowered rapidly in a salt ice bath to 5°. The thick suspension is centrifuged in Servall centrifuges at 15,000 × *g* for 10 minutes, and the resulting supernatant fluid filtered through Whatman No. 1 filter paper. Lyophilization of this slightly opalescent fluid containing the reductase yields 12 to 16 gm. of cream-colored powder from a total of thirty rabbit livers. It can be stored in this form at −20° with no appreciable loss in activity for at least several months.

Further Purification—Two preparations were carried to further stages of purification. In Preparation F-1, 3.5 gm. of lyophilized alcohol extract were dissolved in 10 ml. of 0.1 M Tris, pH 7.30, and centrifuged at 90,000 × *g* for 1 hour. The clear supernatant fluid was dialyzed against 0.1 M Tris, pH 7.30, and then subjected to electrophoresis in a 13 ml. Tiselius cell for two 24 hour periods. A 10 ma. current was employed with intermittent compensation of the boundaries. The ascending side, which moved to the anode, was arbitrarily sampled at three intervals and assayed. The component containing the highest specific activity was diluted to 200 ml. and used as Preparation F-1. Preparation F-2 was made as follows: (*a*) 13 gm. of alcohol extract powder were dissolved in a total volume of 43 ml. with 0.1 M Tris, pH 7.5; (*b*) the protein fraction precipitated between 50 and 80 per cent saturation of ammonium sulfate was obtained; (*c*) precipitate (*b*) was dissolved in 13 ml. of 0.1 M Tris, pH 7.5, and dialyzed against 0.1 M Tris, pH 7.15; (*d*) the dialyzed precipitate was centrifuged at 90,000 × *g* for 1 hour, and the clear supernatant fluid was subjected to electrophoresis at 11 ma. for 26 hours; (*e*) the top 5 cm. of solution at the anode side, containing the reductase of highest specific activity, were drawn off and again fractionated with ammonium sulfate to obtain the precipitate from 50 to 70 per cent saturation. This precipitate was dissolved in 20 ml. of 0.1 M

Tris, pH 7.5. A summary of the specific activities and recoveries with these preparations is presented in Table II. All the preparations were stored at −20° for at least 1 month without appreciable loss of activity.

TABLE II

Summary of Reductase Preparations

Preparation	Per cent recovery	Specific activity,* micromoles cytochrome reduced per min. / Per mg. of material
Lyophilized alcohol extract	100 (Assumed)	0.08
		Per mg. of protein
F-1	40	0.35
F-2	30	4.3

* Assay system: aerobic; temperature 20°; 0.1 μmole of cytochrome *c*, 0.01 μmole of microsomal cytochrome, 0.2 μmole of DPNH; buffer, 0.1 M Tris containing 0.001 M Versene, pH 8.0; volume, 1.00 ml.

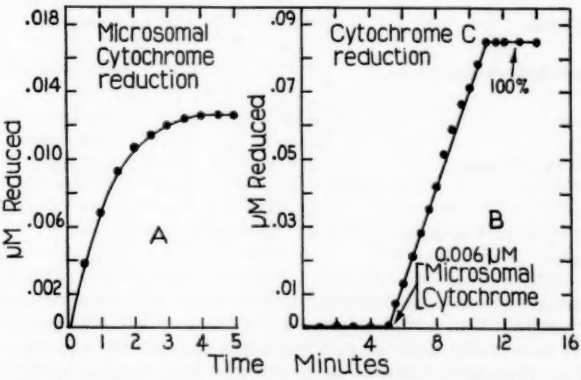

FIG. 1. *A*, reduction of microsomal cytochrome. System: 0.02 ml. of microsomal cytochrome, 0.014 μmole; 0.02 ml. of reductase, Preparation F-1; 0.02 ml. of DPNH, 0.02 μmole; 1.76 ml. of 0.1 M Tris plus 0.001 M Versene, pH 8.0; anaerobic conditions, 20°. *B*, reduction of cytochrome *c*. System: 0.10 ml. of cytochrome *c*, 0.085 μmole; 0.04 ml. of reductase, Preparation F-1; 0.78 ml. of Tris, 0.1 M, pH 8.0; 0.02 ml. of DPNH, 0.2 μmole; aerobic conditions, 20°. After 5 minutes 0.006 μmole of microsomal cytochrome was added.

Properties of DPNH-Microsomal Cytochrome Reductase

Specificity; Electron Donors—The reductase system is specific for DPNH; no appreciable reduction of microsomal cytochrome occurs when TPNH is

added to the reductase as electron donor. Succinate is also totally inactive with this enzyme.

Specificity; Electron Acceptors—The DPNH enzyme shows a second high degree of specificity in that it will catalyze the reduction of microsomal

TABLE III

Activity of DPNH-Microsomal Cytochrome Reductase with Various Acceptors in Place of Cytochrome

Acceptor	Concentration		Condition*	Relative activity
	Of acceptor	Of DPNH		
	M	M		
Indigo monosulfonate	3.3×10^{-3}	7×10^{-5}	Anaerobic	0
" disulfonate	3.3×10^{-3}	7×10^{-5}	Anaerobic	0
" trisulfonate	3.3×10^{-3}	7×10^{-5}	Anaerobic	13
" tetrasulfonate	3.3×10^{-3}	7×10^{-5}	Anaerobic	28
Methylene blue	3.3×10^{-3}	7×10^{-5}	Anaerobic	0
2,6-Dichlorophenolindophenol	3.3×10^{-3}	7×10^{-5}	Anaerobic	18
Ferricyanide	5×10^{-4} to 10^{-6}	1×10^{-4} to 10^{-6}	Aerobic or anaerobic	100
Microsomal cytochrome	1×10^{-5}	5×10^{-5} to 10^{-6}	Anaerobic	13
			Aerobic	11
Cytochrome *c*	1×10^{-4}	1×10^{-4}	"	0
" " + 10^{-5} M microsomal cytochrome	1×10^{-4}	1×10^{-4}	"	23

* The assays were run at 22–25° in 0.1 M Tris buffer or 0.1 M Tris plus 0.001 M Versene at pH 8.0. Two reductase preparations were used in amounts which gave conveniently measurable rates for each substance tested as an acceptor. In each case assays were run under identical conditions with ferricyanide as acceptor to provide a point of reference.

cytochrome but is totally inactive towards cytochrome *c*. The reduction of microsomal cytochrome in an anaerobic system is shown in Fig. 1, *A*. In contrast (Fig. 1, *B*), no reduction of cytochrome *c* occurs during 5 minutes of incubation with DPNH and the reductase. Rapid and complete reduction of cytochrome *c* occurs, however, when catalytic amounts of the microsomal cytochrome are added. The microsomal cytochrome thus acts here as an electron carrier between DPNH and cytochrome *c*. Such systems are used in many of the remaining experiments since they provide a

quick, aerobic method that requires only small amounts of the microsomal cytochrome and accurately measures the activity of the reductase for the microsomal cytochrome. A comparison of the relative rates of reduction in Fig. 1, *A* and Fig. 1, *B* cannot be made because the concentrations of the reacting substances were varied in these experiments.

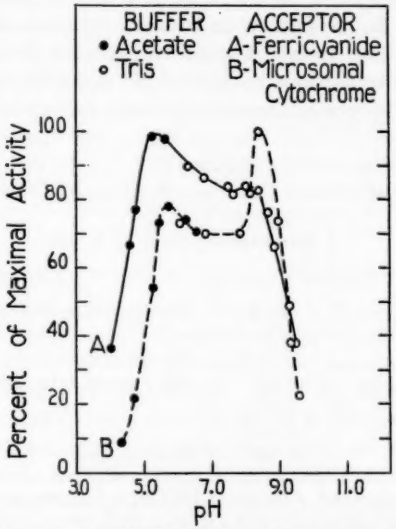

FIG. 2. pH optimum of the DPNH-microsomal cytochrome reductase. All the measurements were made aerobically at 25° in 0.1 M acetate or Tris buffers containing 0.001 M Versene. Final volumes were 1.0 ml., and each test solution contained 0.25 μmole of DPNH and 0.01 ml. of reductase, Preparation F-1. *A*, 0.3 μmole of potassium ferricyanide as final acceptor. *B*, 0.02 μmole of microsomal cytochrome and 0.1 μmole of cytochrome *c* as accepting system. As shown in Table III, the relative rate of ferricyanide to microsomal cytochrome reduction at pH 8.0 and at equivalent concentrations is approximately 5:1.

Table III summarizes the activity of the DPNH dehydrogenase for a number of acceptor compounds. The enzyme, under the assay conditions, is most active with ferricyanide as acceptor. Ferricyanide is reduced from four to eight times more rapidly than microsomal cytochrome, thio indigo tetrasulfonate, or 2,6-dichlorophenolindophenol. While the reductase is active with both 2,6-dichlorophenolindophenol and ferricyanide, it was quite inactive as a catalyst for the reduction of three different preparations of methylene blue.

pH Optima—The pH activity curve for DPNH reductase with either ferricyanide or microsomal cytochrome as acceptor appears in Fig. 2. Fer-

ricyanide is reduced at a maximal rate at a quite acid pH, approximately 5.3, but shows a rather broad optimal pH range from 5.0 to 8.5. The rate of microsomal cytochrome reduction, assayed here with cytochrome *c* as the final electron acceptor, while maximal at pH 8.5, is also high over a rather broad region from pH 5.5 to 9.0.

Flavin Content—Analyses of Preparation F-2 of the reductase for flavin by the method of Burch, Bessey, and Lowry (11) were carried out by Dr. Helen B. Burch. There were 0.4×10^{-3} micromoles of total flavin per mg. of protein. If the total flavin content of the still crude preparation functioned as a prosthetic group, the activity would correspond to 11,000 moles

TABLE IV

Inhibition of Microsomal Cytochrome Reductase by Chelating Agents

Chelating agent	Concentration	Acceptor	Per cent inhibition
	M		
Versene	0.001	Microsomal cytochrome*	0
"	0.10	" "	74
"	0.10	Ferricyanide	0
Sodium pyrophosphate	0.004	Microsomal cytochrome*	41
" "	0.008	" "	63
" "	0.010	" "	66
" "	0.010	Ferricyanide	0

Assay system: aerobic, 20°; 0.02 ml. of DPNH, 0.2 μmole; 0.04 ml. of reductase, Preparation F-1; Tris buffer, pH 8.0, 0.1 M, to a final 1.00 ml.; where indicated, 0.10 ml. of cytochrome *c*, 0.1 μmole and 0.10 ml. of microsomal cytochrome, 0.01 μmole or 0.09 ml. of 0.01 M potassium ferricyanide were added.

* A small amount of the microsomal cytochrome was added as initial acceptor from the reductase and allowed to transfer electrons to a larger amount of cytochrome *c* for optical measurement.

of microsomal cytochrome reduced per minute per mole of flavin. However, no direct evidence for the involvement of flavin in this reductase system has yet been obtained.

Effect of Antimycin A and Dinitrophenol—Antimycin A at concentrations of 10 to 50 γ per ml. had no effect either on the rate of microsomal cytochrome reduction anaerobically or on microsomal cytochrome reduction aerobically with cytochrome *c* as final electron acceptor. Dinitrophenol also had no effect on reductase activity at a concentration of 10^{-3} to 10^{-5} M.

Sulfhydryl Inhibition—The reduction of both ferricyanide and microsomal cytochrome by DPNH and reductase is completely inhibited by 10^{-5} to 10^{-6} M PCMBS.

Effect of Chelating Agents—The effects of Versene and sodium pyrophosphate on the DPNH reductase are summarized in Table IV. Neither re-

agent has an effect on the rate of ferricyanide reduction at concentrations which do inhibit systems containing microsomal cytochrome as initial acceptor and cytochrome *c* as the final acceptor. The inhibition by chelating agents is an effect on electron transfer from reductase to microsomal cytochrome and not on microsomal cytochrome to cytochrome *c* transfer. The oxidation of microsomal cytochrome by cytochrome *c* is not affected by either 0.1 M Versene or 0.1 M sodium pyrophosphate. Low concentrations of Versene (10 to 3 M) also do not inhibit microsomal cytochrome reduction; therefore, this concentration of chelating agent was added to many assay systems to protect the very sensitive sulfhydryl groups on the reductase from any possible heavy metal inactivation. The DPNH-microsomal cytochrome reductase was inhibited by relatively lower concentrations of pyrophosphate and 0.1 M Versene.

DISCUSSION

The large fraction of DPNH-cytochrome *c* reductase activity of liver which is contained in the microsome fraction (2) is shown by the present work to consist in part of a DPNH-specific dehydrogenase which, although totally inactive with cytochrome *c*, catalyzes the transfer of electrons from DPNH to the microsomal cytochrome. Since the latter cytochrome reacts directly with cytochrome *c*, it completes a DPNH-cytochrome *c* reductase system by acting as an intermediate electron carrier. The existence of cytochrome specificities of this type, implicit in current concepts of cytochrome action, had hitherto not been established experimentally.

The properties of the DPNH-microsomal cytochrome reductase, unique in terms of the cytochrome specificity, resemble in some respects those of the DPNH-cytochrome *c* reductase of heart sarcosomes (10, 12–14), although there is by no means a complete correspondence in properties. This is shown by the following comparisons: (*a*) DPNH serves as substrate for both enzymes while TPNH is totally inactive. (*b*) The heart enzyme does not reduce the microsomal cytochrome but reduces cytochrome *c* rapidly in the absence of added cofactors. The microsomal enzyme does not react with cytochrome *c* but reduces the microsomal cytochrome. (*c*) Both enzyme preparations catalyze the reduction of 2,6-dichlorophenolindophenol, exhibiting the so called diaphorase activity. In addition the microsomal cytochrome reductase shows a high activity with ferricyanide as acceptor, an activity not shown by the heart enzyme. (*d*) In contrast to the rather sharp pH optimum of cytochrome *c* reductase with either 2,6-dichlorophenolindophenol or cytochrome *c* as acceptor, microsomal cytochrome reductase has a quite broad pH optimum when either ferricyanide or microsomal cytochrome is reduced. It may be especially significant here that the microsomal cytochrome reductase is a cruder preparation. (*e*) The reductases are similar in their high degree of sensitivity to sulf-

hydryl reagents. (*f*) The cytochrome *c* reductase of heart is a flavoprotein. The microsomal cytochrome reductase preparation contains flavin but it has not yet been possible to demonstrate the direct participation of flavin in the reductase system. (*g*) Finally, microsomal cytochrome reductase shows the same extreme sensitivity to chelating agents that is exhibited by cytochrome *c* reductase. In the case of cytochrome *c* reductase, analysis of the enzyme for iron and experiments demonstrating the partial reactivation of acid preparations by ferric chloride have led to a proposed mechanism for this enzyme involving a DPNH to flavin to metal to cytochrome electron transfer system (14, 15).

SUMMARY

1. The various cytochrome reductase activities observed in microsome suspensions have been obtained in soluble protein fractions.

2. A reductase specific for both DPNH and for microsomal cytochrome has been isolated and found to be completely inactive with TPNH as electron donor or with cytochrome *c* as electron acceptor. Since microsomal cytochrome is rapidly reoxidized by cytochrome *c*, the microsomal cytochrome completes a DPNH-cytochrome *c* reductase system involving a cytochrome to cytochrome electron transfer.

3. The reductase also catalyzes the reduction of ferricyanide, indigo tri- and tetrasulfonates, and 2,6-dichlorophenolindophenol.

4. The reduction of both ferricyanide and microsomal cytochrome is completely inhibited by 10^{-5} M PCMBS.

5. While low concentrations of chelating agents inhibit microsomal cytochrome reduction, the rate of ferricyanide reduction is unaffected by these reagents.

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THE INFLUENCE OF pH ON THE KINETIC CONSTANTS OF α-CHYMOTRYPSIN-CATALYZED ESTEROLYSIS*

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Determination and comparisons of the kinetic constants for the reactions between α-chymotrypsin and various simple synthetic substrates have been extensively made. Several improvements in the mathematical interpretation of such reactions have resulted, and valuable insights into the molecular basis of enzyme specificity have been obtained (1–6). The wide usefulness of α-chymotrypsin in such studies is attributable to its availability, stability, and apparent purity. Recent investigations (7, 8), however, have raised doubts as to the molecular homogeneity of preparations of α-chymotrypsin. We have recently reported[1] briefly on a kinetic investigation into this problem. The present paper presents a more detailed discussion of these studies and includes the results of the extension of these methods to closely related systems. These studies were undertaken in the belief that knowledge of the pH-dependence of the catalytic functions of the enzyme might contribute to the understanding of the relationship between molecular heterogeneity and enzymatic activity. In addition, the quantitative description of pH-dependence in terms of the fundamental kinetic constants of an enzymatic reaction, such as has recently been reported by Gutfreund (9) for trypsin, should be of value to an understanding of the reaction mechanism.

EXPERIMENTAL

Materials—The α-,[2] β-, and γ-chymotrypsin preparations used in these investigations were prepared by the ammonium sulfate fractionation procedure of Northrop *et al.* (10) and were stored in the cold as salt-free, lyophilized powders. Each day, fresh enzyme was dissolved in 0.001 N HCl and stored at 5–10° for use as a stock enzyme solution. All enzyme concentrations were determined spectrophotometrically at 282 mμ, by using the relation, mg. of N per ml. = 0.0763 × optical density (7).

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[1] A preliminary report of this work was presented before the Forty-sixth meeting of the American Society of Biological Chemists at San Francisco, April, 1955 (1).

[2] Obtained through the generosity of Dr. Jules A. Gladner and Dr. Hans Neurath.

Acetyl-L-tryptophan[3] and acetyl-L-tryptophan ethyl ester were commercial products (Mann Research Laboratories). All preparations of ATrEE that were used in experiments reported here were shown by paper chromatography to be free of contamination with ATr. Acetyl-L-tyrosine ethyl ester was prepared according to a published procedure (11). Stock solutions of 0.0100 M ATEE and 0.0067 M ATrEE in water could be prepared by warming suspensions of these compounds to 85°. Upon rapid cooling to room temperature, clear supersaturated solutions of these slightly soluble compounds were obtained which were stable for periods up to 12 hours. Concentrations of these solutions calculated from maximal enzymatic release of hydrogen ions agreed well with the weight concentrations.

All other materials were of reagent grade.

Methods—The usual continuous potentiometric titration method for following esterolytic reactions was employed (7, 12). The assay mixture consisted usually of 10 ml. of substrate, 0.5 ml. of 2.4 M $CaCl_2$, 0.5 ml. of 0.08 M buffer, and 1.0 ml. of enzyme solution. Thus, the final concentration of $CaCl_2$ was 0.1 M. The dependence of k_3 and of an apparent K_s on the ionic strength of the assay mixture has been noted by several investigators (13, 14). The use of $CaCl_2$ in the concentration mentioned is sufficient to prevent erratic results from variations in ionic strength owing to the addition to the reaction mixture of other ionic components such as competitive inhibitors, and to give near maximal values for k_3. When the volume of substrate added was varied, the total volume was brought to 12 ml. by the addition of distilled water. The standard base used for titration was usually 0.1 N NaOH, but 0.02 N NaOH was employed in runs in which the substrate concentrations were too low for use of the more concentrated base.

All pH measurements were made with a Cambridge model R pH meter standardized at pH 4 with 0.05 M potassium acid phthalate.

Results

Determination of Kinetic Constants of ATrEE at pH 8—Measurements of the initial velocity of the α-chymotrypsin-catalyzed hydrolysis of ATrEE were made at several substrate concentrations and at pH 8. The values obtained in one such experiment are plotted in Fig. 1 in the manner of Lineweaver and Burk (15). From the slopes and intercepts, K_s may be calculated to be $9.3 \times 10^{-5} \pm 2.0$ moles liter^{-1}. Determination of a K_s this small required the use of 0.02 N NaOH as the titration base and represents very nearly the extreme experimental limit of the esterase procedure

[3] The following abbreviations are used: ATr, acetyl-L-tryptophan; ATrEE, acetyl-L-tryptophan ethyl ester; ATEE, acetyl-L-tyrosine ethyl ester; Tris, tris(hydroxymethyl)aminomethane; $ATrNH_2$, acetyl-L-tryptophanamide; $ATNH_2$, acetyl-L-tyrosinamide.

employed. As will be evident from other data (see below), K_s for ATrEE falls almost within the experimental error of the method as it was routinely used for pH-dependence studies.

Determination of pH-Dependence of k_3 of ATrEE—Measurements of the entire course of the α-chymotrypsin-catalyzed hydrolysis of ATrEE were made at varying values of pH. Three buffers, Tris-HCl, imidazole-HCl, and cacodylic acid-NaOH were required to cover the desired range. None of these buffers appeared to exert any specific influence on the course of the reaction. In these studies of pH-dependence the concentration of ATrEE was held constant near 0.0083 M, but three concentrations of

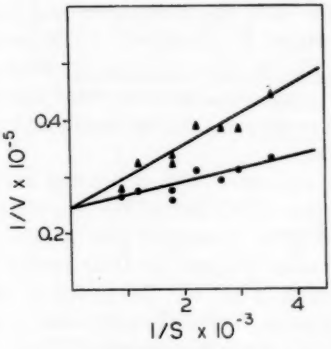

FIG. 1

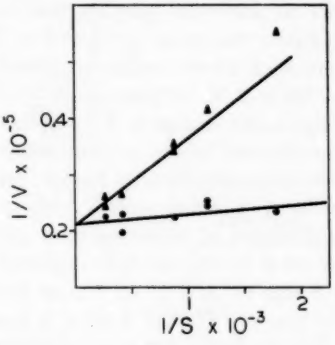

FIG. 2

FIG. 1. Effect of initial concentration of ATrEE and of ATr on the initial velocity of the α-chymotrypsin-catalyzed hydrolysis. ●, ATrEE alone; ▲, ATrEE + 0.0125 M ATr. T 25°; buffer, Tris-HCl; pH 8.0.

FIG. 2. Effect of initial concentration of ATrEE and of ATr on the initial velocity of the α-chymotrypsin-catalyzed hydrolysis. ●, ATrEE alone; ▲, ATrEE + 0.0125 M ATr. T 25°; buffer, cacodylic acid-NaOH; pH 6.1.

enzyme were required so that reasonable reaction rates might be obtained. The enzyme concentration in runs above pH 7.5 was near 7.45×10^{-4} mg. N ml.$^{-1}$, and the buffer was commonly Tris, while in runs between pH 6.5 and 7.5 the enzyme concentration was near 1.49×10^{-3} mg. N ml.$^{-1}$, and the buffer was imidazole. Below pH 6.5 the enzyme concentration was about 3.75×10^{-3} mg. N ml.$^{-1}$, and the buffer was cacodylic acid. In all cases the buffer and enzyme concentration regions were overlapped so as to disclose any specific effects attributable to buffer or enzyme concentration. The data obtained were plotted according to the integrated form of the Michaelis-Menten equation (16)

$$k_3et = 2.3K_s \log \frac{a_0}{a} + (a_0 - a) \qquad (1)$$

where a_0 = initial substrate concentration, a = substrate concentration at time t, e = enzyme concentration, K_s = the Michaelis constant, and k_3 = the rate constant for the decomposition of the enzyme substrate complex into products and enzyme. The calculations make use of the previously determined value of K_s. As already indicated, the contribution of the first order term in Equation 1 for our usual system is quite small, since K_s is so low. Nevertheless, its use was necessary, and sufficient, to obtain a straight line plot at longer reaction times at pH values above 7 to 7.5. Thus, there was no indication of product inhibition in this region. Below pH 7, however, a progressive deviation from linearity with decreasing pH was observed, indicating a possible variation in K_s for the system. In order to determine whether this was the case, the Lineweaver and Burk procedure was again employed to determine K_s at pH 6.1. The results of one such determination are shown in Fig. 2. The value for K_s obtained was $8.4 \pm 2 \times 10^{-5}$ mole liter^{-1}. It is apparent, therefore, that there is no significant change in K_s over this pH range and that the kinetic changes detected must be due to some other cause.

The demonstration by Foster and his associates (3–6) of product inhibition by ATr in the system α-chymotrypsin-ATrNH$_2$ and the dependence of the degree of inhibition upon pH led us to investigate this possibility. The value for K_I for ATr reported by these workers for their system at pH 8 was $10 \pm 2 \times 10^{-3}$ mole liter^{-1} and, at pH 6.9, $2.0 \pm 0.3 \times 10^{-3}$ mole liter^{-1}. The pH 8 value is large enough so that the inhibition would normally be below the level of detection in our esterase system. However, by again employing the method of Lineweaver and Burk, K_I for ATr was determined in the ATrEE-α-chymotrypsin system at pH 6.1 and at pH 8. The results of these investigations are also given in Figs. 1 and 2. From these graphs K_I was determined to be $8.7 \pm 2 \times 10^{-3}$ mole liter^{-1} at pH 8 and $1.7 \pm 0.5 \times 10^{-3}$ mole liter^{-1} at pH 6.1. The deviation from linearity in the plot of data at pH 6.1 occurs in the region of the extreme limit of the experimental procedure. This deviation was obtained in every experiment to varying degrees, but the limiting slope of the straight line portion always corresponded to a value of K_I near 1.7×10^{-3} mole liter^{-1}. Since it was apparent that K_s for the enzymatic reaction was constant, but that variation of the degree of inhibition by reaction products was being obtained, the kinetic plot suggested by Foster and Niemann (6) appeared ideal for more complete characterization of the enzymatic reaction. The equation is obtained from the integrated form of the Michaelis-Menten equation for the case of inhibition by one of the reaction products.

$$k_3 et = 2.3K_s\left(1 + \frac{a_0}{K_I}\right)\log\frac{a_0}{a} + \left(1 - \frac{K_s}{K_I}\right)(a_0 - a) \qquad (2)$$

By dividing by t, and rearrangement, the equation becomes

$$\frac{a_0 - a}{t} = \left[\frac{-2.3\,K_s\left(1 + \frac{a_0}{K_I}\right)}{1 - \frac{K_s}{K_I}}\right]\left[\frac{\log \frac{a_0}{a}}{t}\right] + \frac{k_3 e}{1 - \frac{K_s}{K_I}} \qquad (3)$$

where K_I = dissociation constant for the enzyme-inhibitor compound. Thus a plot of $(a_0 - a)/t$ *versus* $(\log a_0/a)/t$ for a single esterase measurement should yield a straight line with a slope equal to $(-2.3K_s(1 + a_0/K_I))/(1 - K_s/K_I)$ and with an intercept equal to $k_3e/(1 - K_s/K_I)$. When the

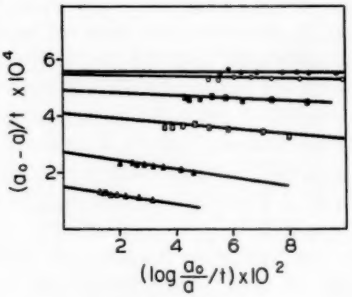

FIG. 3

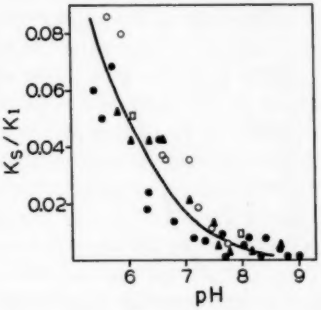

FIG. 4

FIG. 3. The hydrolysis of ATrEE by α-chymotrypsin. pH 8.43, ●; pH 8.02, ○; pH 7.36, ■; pH 6.38, □; pH 5.72, ▲; pH 5.41, △ (see the text).

FIG. 4. The dependence of the ratio of K_s for ATrEE to K_I for ATr on the pH of the system. ●, α-Chymotrypsin; ○, β-chymotrypsin; ▲, γ-chymotrypsin; □, calculated from the values obtained from Figs. 1 and 2. T 25°.

data from the series of experiments at varying pH values were plotted in this fashion, a family of straight lines of negative slope was obtained. An example of these data is shown in Fig. 3. For high pH values the slope is quite small, as is to be expected from the known values of K_s and K_I in this region. As the pH decreases below 6.5, however, there occurs a gradual increase in the negative slope of these lines. By utilizing the equations just described and a value of 9×10^{-5} mole liter^{-1} for K_s, a value of K_s/K_I was determined for each run. These values are shown in Fig. 4. Though there is considerable scatter in these points, since they represent values near the limit of determination in the system employed, it is clear that there is a progressive decrease in K_I as the pH is decreased.

Values of k_3e may be determined for each experiment from the intercepts of the lines on the $a_0 - a/t$ axis. From Fig. 4 it is apparent that $1 - K_s/K_I$ is a negligible correction to the intercept at high pH values and rises only

to 6 to 8 per cent at the lowest pH values investigated. Since e is known, k_3 may be evaluated for each experiment. Values for k_3 had been previously determined from the initial slopes of the plots of the data according to Equation 1 without other regard to product inhibition. These values agreed, as would be predicted, with those obtained in this manner within experimental error, except perhaps at the lowest pH values. When these values for k_3 were plotted against pH, it was apparent that k_3 varied with pH in a manner resembling the titration curve of a weak acid. The maximal value of k_3 was approached in the pH region between 8.3 and 9. At pH 8.7 the experimentally determined value of k_3 was found to be 0.77 ±

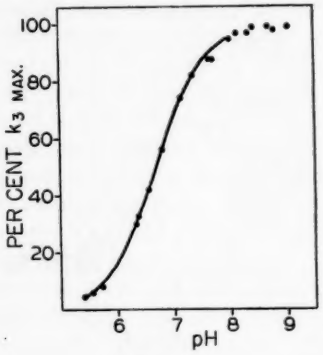

FIG. 5

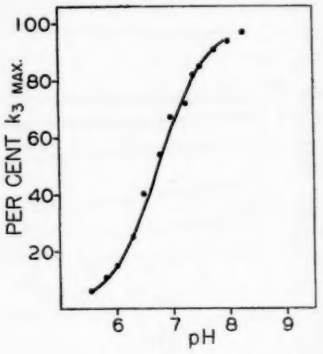

FIG. 6

FIG. 5. The dependence of the degree of esterolytic activity of α-chymotrypsin upon pH. Substrate, ATrEE; T 25°.

FIG. 6. The dependence of the degree of esterolytic activity of α-chymotrypsin upon pH. Substrate, ATEE; T 25°.

0.03 mole liter^{-1} min.$^{-1}$ mg. N^{-1} ml. From the form of the curve, this value was assumed to be 99 per cent of the maximal k_3 of the system. Fig. 5 shows the variation of k_3 with pH calculated on the basis of per cent of maximal k_3 as described. The line through the experimental points represents the theoretical dissociation curve of a weak acid of pK′ = 6.70. Since the substrate, ATrEE, undergoes no change in structure over the pH region investigated, it seems reasonable to ascribe the behavior observed to the enzyme.

Another series of reactions was studied at varying pH levels in which all the conditions were the same as those described above, except that the temperature was maintained at 14° instead of 25°. The resulting decrease in rates required that all enzyme concentrations be approximately doubled. Again, the data were examined as a function of pH and the highest value of k_3, 0.37 ± 0.02 mole liter^{-1} min.$^{-1}$ mg. N^{-1} ml., was set as being 99 per

cent of the maximal theoretical value. In this case the experimental data are best described in terms of the dissociation constant of a weak acid of $pK' = 7.00$. Although the scatter is somewhat more than was obtained in the previous case, there is no doubt that the entire curve has been displaced to higher pH values, as would be expected if the variation does represent a pH-dependent change in ionic form of the enzyme.

Determination of pH-Dependence of k_3 of ATEE—A series of esterase assays was performed which differed from previous determinations in that ATEE was used as substrate. The reactions were studied at 25° with the use of lower concentrations of enzyme, since k_3 for ATEE was known (2) to be higher than for ATrEE. In a similar system, k_s for ATEE was reported (7) to be 7×10^{-4} mole liter^{-1}. Upon reexamination in the system employed for these measurements, K_s was again found to be $7.0 \pm 0.5 \times 10^{-4}$ mole liter^{-1} at pH 8.0. Since this value of K_s was sufficient to yield a straight line plot above pH 6.5 when employed with the data from these experiments in Equation 1, it appeared justifiable on the basis of the experience with ATrEE just outlined to determine k_3 directly from the initial slope of this type of plot. This is further strengthened by consideration of the K_s/K_I ratios calculated for $ATrNH_2$, 0.5, and $ATNH_2$, 0.3 at pH 7.9 (3). As a result of several measurements of k_3 of ATEE in the pH region 8 to 9, the maximal value of k_3 for this substrate was set at 2.87 ± 0.10 mole liter^{-1} min.$^{-1}$ mg. N ml.$^{-1}$. On this basis the values obtained in this pH-dependence series are shown in Fig. 6. In this case the theoretical line required a pK' of 6.74 for the best fit of the data. This is close to the value of 6.70 determined for ATrEE, the difference presumably being due to small errors in setting limits for the theoretical lines and to the expected experimental error.

Comparison of Other Chymotrypsins—Since two other forms of chymotrypsin, β-chymotrypsin and γ-chymotrypsin, have been isolated from the supernatant solution of crystalline α-chymotrypsin preparations (10), it appeared of importance to characterize the pH-dependence of these enzymes. A series of runs at varying pH values was made with each enzyme with ATrEE as substrate. The data were plotted according to Equation 3. Since previous investigators (10) have found little or no evidence that the kinetic constants of these enzymes differ from those of the α form, the value of K_s obtained for ATrEE with α-chymotrypsin was used to calculate K_s/K_I for these enzymes. The data are shown in Fig. 4. It may be seen that K_I varies in approximately the same way for all three forms of chymotrypsin. Values of k_3 were calculated from the intercepts as before and plotted as per cent of maximal k_3 in Fig. 7. The pK' value producing the best fit to the experimental data was 6.75 for γ-chymotrypsin and 6.67 for β-chymotrypsin. The maximal value of k_3 for γ-chymotrypsin was

determined to be 0.78 ± 0.04 mole liter^{-1} min.$^{-1}$ mg. of N^{-1} ml., which is identical within experimental error with that obtained for α-chymotrypsin. For β-chymotrypsin a value of 0.53 ± 0.03 mole liter^{-1} min.$^{-1}$ mg. of N^{-1} ml. was obtained for k_3 maximum. Unfortunately, this preparation appeared to contain amorphous material which could not be removed easily by crystallization. Thus, the low value obtained for k_3 is

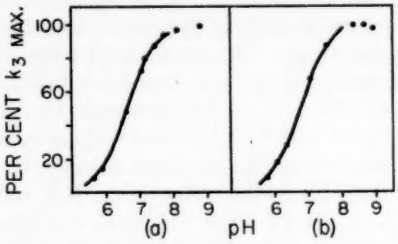

FIG. 7. The dependence of the degree of esterolytic activity of β- and γ-chymotrypsin upon pH. Substrate, ATrEE; T 25°. *a*, β-chymotrypsin; *b*, γ-chymotrypsin.

TABLE I

Summary of pH-Dependence Experiments

Enzyme	Substrate	Temperature	pK′	K_s	k_{3max}	k_1'*
		°C.		*mole l.*$^{-1}$	*mole l.*$^{-1}$ *min.*$^{-1}$ *mg. N*$^{-1}$ *ml.*	*mole l.*$^{-1}$ *min.*$^{-1}$ *mg. N*$^{-1}$ *ml.*
α-Cht†	ATrEE	25	6.70 ± 0.03	8.9 ± 2 × 10^{-5}	0.78 ± 0.03	0.76 ± 0.04
"	"	14	7.00 ± 0.05	(9 × 10^{-5})‡	0.38 ± 0.02	0.36 ± 0.03
"	ATEE	25	6.74 ± 0.05	7.0 ± 0.5 × 10^{-4}	2.87 ± 0.10	2.78 ± 0.1
β-Cht	ATrEE	25	6.67 ± 0.05	(9 × 10^{-5})‡	0.53 ± 0.03	0.51 ± 0.04
γ-Cht	"	25	6.75 ± 0.05	(9 × 10^{-5})‡	0.78 ± 0.04	0.76 ± 0.05

* The average value of k_3 between pH 8.3 and 8.5.

† Cht is used as a contraction for chymotrypsin.

‡ Assumed from the value for α-chymotrypsin and ATrEE at 25°.

probably due to the presence of inactive protein. The comparison of rates of change of activity with pH remains valid, however, since it is made on the basis of per cent of maximal k_3 for each enzyme. The various values determined for the pK′ producing the best fit to the experimental data are collected in Table I. It may be seen that a value of 6.71 ± 0.05 would satisfactorily describe all the data, within the limits of experimental variation, except for the experiment carried out at 14°.

Comparison of α-Chymotrypsin Preparations—The question of the variability of samples of α-chymotrypsin prepared according to various pro-

cedures or obtained commercially has been a matter of some concern. To test the reliability of enzyme preparations obtained by different methods, a comparison was made between the crystalline α-chymotrypsin used in all the other experiments, the same preparation after two alcohol recrystallizations (17), and a commercially obtained alcohol recrystallized preparation. Table II shows the comparison of these samples at pH 8. It is apparent that the commercial preparation is only about 92 per cent as active as the other preparations. Such agreement appears remarkable in view of our current understanding of the complex nature of the chymotrypsinogen activation process (18).

TABLE II

Comparison of α-Chymotrypsin Preparations

Enzyme	Substrate	k_3
		mole $l.^{-1}$ *min.*$^{-1}$ *mg. N*$^{-1}$ *ml.*
Salt fractionation (Kunitz)	ATrEE	0.72
	ATEE	2.67
Same + 2 alcohol recrystallizations	ATrEE	0.72
	ATEE	2.70
Commercial alcohol recrystallized	ATrEE	0.66
	ATEE	2.50

DISCUSSION

The chymotrypsin substrate employed in the majority of investigations reported here was ATrEE. As this compound exists in solution in only one form over the pH region investigated, any pH-dependent variation observed in this enzyme substrate system must be ascribed to changes in the enzyme. The excellent agreement obtained between experimental data describing the variation in k_3 for the α-chymotrypsin-catalyzed hydrolysis of ATrEE with pH and a theoretical titration curve would seem to justify two conclusions. The first is that the loss of a proton from some weakly acidic group in the enzyme, pK′ 6.7, is necessary before enzymatic activity is detectable. It seems reasonable to assume that k_3 as defined in the current formulation of the Michaelis-Menten theory (2) for this system actually does not change. Rather, the change in the rate of the reaction with pH is due to the variation of the amount of the enzyme present in the catalytically active form. This is further substantiated by the close agreement of the activity *versus* the pH curve for another substrate, ATEE, with that obtained for ATrEE.

The second conclusion we may draw from this agreement is that, within

the limits of these determinations, there is no evidence for heterogeneity of the enzyme preparation. Thus, whatever the cause of the heterogeneous appearance of α-chymotrypsin in electrophoresis (8), it appears that the ionization constant of a functional group critical for enzymatic activity is the same for all of the components. This interpretation is supported by the results obtained with β- and γ-chymotrypsin. Within the limits of measurement these two forms of chymotrypsin are kinetically identical to the α form. Chemical studies of these proteins have also indicated a close similarity to the α form of the enzyme. The *N*- and *C*-terminal residues are all identical, but the β and γ forms yield a greater variety of fractional amounts of amino acid per mole of protein when treated with carboxypeptidase (18). Thus, an unexplained difference in solubility of the various preparations remains the chief distinguishing characteristic (10). Whatever the structural changes involved, it is apparent that they do not affect, and thus presumably do not involve, the portion of the molecule concerned with the specific catalytic activity.

The shift in the pH-activity curve which was obtained on lowering the temperature is characteristic of the acidic groups in proteins known to ionize in this pH region (19). By utilizing the values for $-\log K'$ at 14°, 7.00, and at 25°, 6.70, for the reaction between α-chymotrypsin and ATrEE, a value for ΔH, the heat of ionization, for this functional group associated with enzymatic activity may be calculated to be approximately 11 kilocalorie mole^{-1}. Gutfreund (9), working with the trypsin-benzoyl-L-arginine-ethyl ester system, has determined the pK′ for trypsin[4] to be 6.25 at 25° and the heat of ionization to be 7 kilocalorie mole^{-1}. The agreement of these values with those reported for imidazole groups in proteins (19) has led him to suggest that a histidine residue in the trypsin molecule is directly concerned with the catalytic activity of the protein. In α-chymotrypsin the pK′ associated with enzymatic activity also lies within the range associated with the imidazole group, but the value determined for ΔH more closely corresponds to those determined for amino groups. However, it appears unlikely that terminal amino groups have pK′ values below 7.4 (19). Chemical analysis of the protein (10) has failed to provide evidence for any type of non-amino acid prosthetic group which might give rise to the observed variation with pH. Obviously any postulates based on these facts overlook the possibility that the specific spatial location of the group in the enzyme molecule may have greatly changed its acidic character from that usually encountered. Further speculations as to the nature of this group or groups in these closely related proteolytic enzymes must therefore await the availability of other types of information.

[4] In a brief study of this system we have determined the pK′ for trypsin activity to be about 6.40. Our system, however, contained 0.10 M $CaCl_2$ to stabilize the enzyme.

The variation of K_I for ATr with pH must also be considered in a discussion of the nature of the ionizable group necessary for enzymatic activity. The suggestion that there is a negatively charged group in or near the active center of chymotrypsin was first made by Neurath and Schwert (2) on the basis of kinetic studies of anionic and neutral specific inhibitors. Recently, Foster and Niemann (5) reinvestigated this point, utilizing a series of more closely related inhibitors. In measurements at pH 6.9 and 7.9, they detected a variation in K_I for anionic inhibitors such as ATr, but could find no significant change for neutral inhibitors such as acetyl-D-tryptophanamide. On the basis of these and other comparative studies, they concluded that in or near the active center of the enzyme there is at least one negatively charged group. This group is postulated to be chiefly in the conjugate acid form at pH 6.9 and much less so at 7.8. The results we have obtained for K_I for ATr in the esterase system are generally in accord with such a hypothesis. However, the scope of the determinations is insufficient to warrant any quantitative statement concerning the actual degree of protonation at any pH, except that it is apparently close to 100 per cent near pH 5.5. The pK′ for the ionization of ATr is far too low to consider the variation of the COO/COOH ratio of this compound as a major factor in the pH-dependence of the affinity of the enzyme for ATr in the region above pH 5.

A similar variation in the degree of binding was found by Doherty and Vaslow (20) in their study of the thermodynamics of the interaction between acetyl-3,5,-dibromo-L-tyrosine and chymotrypsin. These workers were considering this compound as a substrate for the enzyme, whereas in our measurements the interaction between ATr and the enzyme was measured as competitive inhibition. It seems probable that the physiological hydrolytic function of this enzyme is dependent on the fact that the interaction between the protein- and carboxyl-substituted substrates remains constant as the pH is increased toward the optimum, while the interaction between enzyme- and carboxyl-unsubstituted "substrates" declines. The nature of the negatively charged group in the active center is unknown. Its relationship to the group of pK′ 6.7 which controls the catalytic function of the enzyme is likewise uncertain.

It may be seen that all three chymotrypsins are approximately 95 per cent in the catalytically active form at pH 8 and that the rate of increase with pH toward 100 per cent is small between pH 8 and 9. Extensive investigations were not carried out above pH 9 owing to the uncertainties introduced by the instability of the enzyme and substrate and the titrimetric difficulties common to the high pH region. The presence of high concentrations of calcium ions further contributed to the decreasing effectiveness of the esterase method at high pH. In a few exploratory experiments, however, some evidence was obtained which indicated that the

apparent K_s of the reaction begins to increase rapidly above pH 9. It is difficult, therefore, to set a pH optimum for the enzymes and the two ester substrates which were used. For operational purposes the pH optimum is probably best defined in this case as the pH region from 8 to 8.5, where the apparent k_3 is from 95 to 98 per cent of the maximal k_3. In this region the α-chymotrypsin-catalyzed hydrolysis of ATrEE approaches zero order kinetics at substrate concentrations below 0.01 M. Under these conditions, therefore, this substrate would appear to be nearly ideal for routine assay of α-chymotrypsin.

The assistance of Dr. James E. Hurley and Mr. E. K. Carney in the performance of some of the initial measurements is gratefully acknowledged.

SUMMARY

The action of α-, β-, and γ-chymotrypsin preparations on two synthetic substrates, ATrEE and ATEE, has been studied as a function of pH. In all cases the variation of the apparent k_3 with pH could be described by the assumption that enzymatic activity of the protein required the loss of a proton from a particular weakly acidic group in the protein. Within the limits of the experiment, the pK′ of this group was found to be the same, 6.71 ± 0.05, in all cases examined. While no evidence could be obtained from these studies which might indicate heterogeneity of the enzyme preparations, the close similarity of behavior of the three forms of chymotrypsin demonstrates the limited sensitivity of this test. Measurement of the change in pK′ with temperature led to a value of ΔH of ionization of 11 ± 2 kilocalorie mole^{-1}. The relationship of these values to those which have been determined for known functional groups in proteins has been discussed.

It has been shown that the apparent K_I for one of the products of the reaction, ATr, is a continuous function of the pH of the system over the range pH 5.5 to 8. Possible explanations for this behavior have been discussed.

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ISOLATION OF RADIOFORMALDEHYDE IN THE METABOLISM OF DIMETHYLAMINOETHANOL-$C^{14}H_3$*

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Several years ago we reported that formaldehyde could be isolated when dimethylaminoethanol, dimethylglycine, sarcosine, or methanol was incubated with a whole liver homogenate (1). Dimethylaminoethanol differed from the other substrates in that it required both the supernatant and sediment fractions of the homogenate to effect this biochemical oxidation. We have now prepared methyl-labeled dimethylaminoethanol and examined its metabolism in the whole liver homogenate preparation.

EXPERIMENTAL

Synthesis of Dimethylaminoethanol-$C^{14}H_3$—5 mmoles of monomethylaminoethanol were refluxed with 15 mmoles of formic acid and 5.54 mmoles of radioformaldehyde[1] containing 3.82×10^7 c.p.m., corrected (2). At the end of 12 hours residual monomethylaminoethanol was converted to the nitroso derivative (3) and the acidified mixture was evaporated to dryness *in vacuo* to remove unreacted formaldehyde and formate. The residue was then dissolved in concentrated NaOH and the dimethylaminoethanol was steam-distilled until the distillate was no longer alkaline.

Since, to our knowledge, there was no way to isolate the dimethylaminoethanol from the distillate as a crystalline product suitable for use in enzyme experiments, we resorted to a combination of several analytical procedures in order to determine the purity of the solution. First, the radiodimethylaminoethanol in an aliquot of the distillate was isolated as the picrolonate (4) by the carrier technique and counted in a thin window Geiger-Müller counter. The derivative melted at 196.5°, uncorrected. The total activity of the dimethylaminoethanol in the distillate was found to be 1.76×10^7 c.p.m., corrected. This was equivalent to the activity of 2.6 mmoles of the starting radioformaldehyde and therefore represented a

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[1] Received on allocation from the United States Atomic Energy Commission.

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yield of 2.6 mmoles of labeled dimethylaminoethanol. Next, the distillate was analyzed by a micro-Kjeldahl procedure and found to contain 2.6 mmoles of N. Finally, electrometric titration of the distillate showed the presence of 2.6 mmoles of a base with a pK identical with that obtained with an authentic sample of dimethylaminoethanol. Thus, the only basic and the only nitrogenous compound in the solution was radiodimethylaminoethanol. The specific activity of the labeled dimethylaminoethanol was 6.8×10^6 c.p.m. per mmole. Prior to use, this material was diluted to contain 3.1×10^5 c.p.m. per 0.225 mmole, except for the Warburg experiments for which the undiluted material was employed.

Before using the radiodimethylaminoethanol in enzyme experiments, precaution was taken to reduce to a minimum any traces of radioformaldehyde still contaminating the solution. This was accomplished by adding HCl and non-isotopic formaldehyde to the dimethylaminoethanol solution and evaporating to dryness. The residue was then dissolved twice in water and evaporated. When the final residue was redissolved and analyzed by the carrier procedure with ordinary formaldehyde, it was found that the solution contained not more than 1 molecule of radioformaldehyde for every 3×10^4 molecules of radiodimethylaminoethanol·HCl.

Enzyme Experiments—Sprague-Dawley rats, fasted for 16 to 24 hours, were decapitated and their livers were homogenized in the potassium phosphate buffer described previously (1). 20 ml. of the homogenate, containing 2.5 gm. of liver, were placed in a 125 ml. Erlenmeyer flask and adjusted to pH 8.8 with 0.1 N KOH. The contents of the flask were then warmed to 37° in a water bath and 2 ml. of buffer containing 0.225 mmole of radiodimethylaminoethanol adjusted to pH 8.8 were added. The flasks were shaken in a constant temperature room at 37° for 1 hour. At the end of that time proteins were precipitated by the addition of 10 ml. of 20 per cent trichloroacetic acid followed by centrifugation. The precipitate was washed twice with 5 ml. portions of 10 per cent trichloroacetic acid and the washings were added to the original supernatant fluid.

Carrier formaldehyde was added to the trichloroacetic acid solution, reisolated as the dimedon derivative, and plated and counted as described previously (5). The formaldemethone was recrystallized from ethanol and water to a constant specific activity. The derivative melted at 188–189°, uncorrected.

Results

As shown in Table I, the incubation of radiodimethylaminoethanol with the homogenized liver resulted in the formation of radioformaldehyde. When the flask containing the homogenate was immersed in a boiling water bath for 7 minutes prior to the incubation, no radioformaldehyde could be isolated.

Since it had been found previously in this laboratory that semicarbazide traps formaldehyde quantitatively in the oxidation of sarcosine by mitochondria (6), radiodimethylaminoethanol was incubated with homogenized liver in the presence of 0.5 mmole of this reagent. Under these conditions, the yield of radioformaldehyde was increased 3-fold (Table I).

In supplementary Warburg experiments, 22.5 μmoles of undiluted radiodimethylaminoethanol were incubated for 2 hours in an atmosphere of air with the homogenate from 0.5 gm. of liver. At the end of the experiment 3 per cent of the incubated C^{14} was isolated as radioformaldehyde and 70 per cent as the picrolonate of dimethylaminoethanol by the carrier procedure. The reaction flasks contained negligible amounts of $C^{14}O_2$. The increase in oxygen uptake due to the presence of the substrate was 3.1 μmoles,

TABLE I

Isolation of Radioformaldehyde from Dimethylaminoethanol-$C^{14}H_2$ Incubated with Homogenized Liver

0.225 mmole of substrate containing 3.1×10^5 c.p.m. was incubated for 1 hour in air with the homogenate from 2.5 gm. of liver. Radioformaldehyde was isolated as the dimedon derivative. All experiments were in triplicate.

Incubated	Formaldemethone	CH_2O*
	c.p.m.	*μmoles*
Substrate	1270	1.8
" in heated homogenate	0	0
" + semicarbazide	3306	4.8

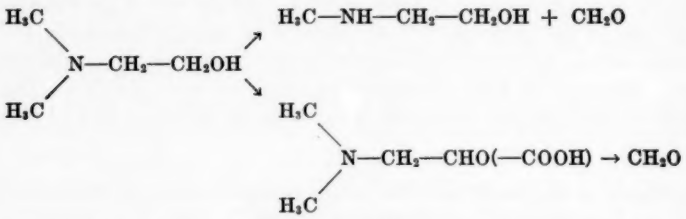
* Since only one of the substrate methyl groups was labeled, the counts per minute was multiplied by 2 in calculating the yield of formaldehyde.

a quantity sufficient to effect the divalent oxidation of 28 per cent of the incubated dimethylaminoethanol. Even if we assume, on the basis of the semicarbazide experiment, that the production of formaldehyde may have been 3 times greater than the quantity isolated, *i.e.* 9 per cent of the incubated C^{14}, an appreciable amount of the products of dimethylaminoethanol metabolism is still unaccounted for.

DISCUSSION

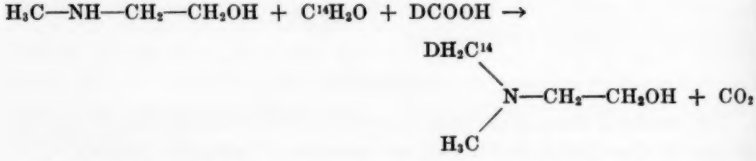
The results of these experiments provide direct evidence that the methyl groups of dimethylaminoethanol are sources of metabolic formaldehyde. Although the exact pathway of the conversion of dimethylaminoethanol to formaldehyde in homogenized liver is not known, it is definitely circumscribed by related experiments (1) in which we have shown that choline and monomethylaminoethanol are not sources of detectable formaldehyde in this preparation. Thus, the formation of these compounds is not a *prerequisite* for the conversion of the methyl carbon of dimethylaminoethanol

to formaldehyde. Furthermore, monomethyl- and dimethylamine are not sources of formaldehyde in liver homogenate. Consequently, it appears either that dimethylaminoethanol is oxidized directly to yield formaldehyde and monomethylaminoethanol, or, alternatively, that its alcohol group is oxidized to yield dimethylaminoacetaldehyde or dimethylglycine prior to the oxidative demethylation. These alternative pathways of metabolism in liver homogenate are illustrated as follows:



Certainly in the intact animal in which dimethylaminoethanol may be converted to dimethylglycine via choline (7, 8) and betaine (9, 10), the dimethylglycine can function as an intermediate in the oxidation of the methyl groups of dimethylaminoethanol to formaldehyde in the "1-carbon cycle" previously formulated (11). However, whether dimethylaminoethanol can be also shunted across the cycle in liver by direct oxidation of its alcohol group to yield dimethylglycine is presently unknown.

Finally, the synthesis and analysis of dimethylaminoethanol-$C^{14}H_3$ described in this paper elucidate certain aspects of the now classical formate-formaldehyde methylation reaction (2). Earlier, du Vigneaud and coworkers (7) showed that in this reaction deuteroformate may be used to synthesize dimethylaminoethanol containing D in the methyl group. We have now shown that only formaldehyde is a source of the methyl carbon, since the specific activities of the formaldehyde and methyl carbons are identical.



The initial step in the above reaction is presumably the condensation of monomethylaminoethanol and formaldehyde, followed by reduction with formate of the formaldehyde carbon to yield the new methyl group. We have discussed elsewhere (11) the likelihood that an analogous methylation reaction involving the synthesis of dimethylaminoethanol from form-

aldehyde and aminoethanol occurs *in vivo*, and that only the addition of the third methyl group to yield choline is restricted to transmethylation with methionine as the methyl donor.

SUMMARY

Dimethylaminoethanol-$C^{14}H_3$ has been synthesized by the classical methylation reaction with monomethylaminoethanol, formic acid, and radioformaldehyde. The specific activity of the product indicates that in this reaction formaldehyde, and not formate, is the sole source of the methyl carbon.

When the radiodimethylaminoethanol was incubated with a whole liver homogenate, radioformaldehyde accumulated and was isolated as the dimedon derivative. Addition of semicarbazide to the incubation mixture increased the yield of radioformaldehyde 3-fold.

The implications of these results are discussed with respect to the pathway of dimethylaminoethanol metabolism.

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RING N METHYLATION; A MAJOR ROUTE OF HISTAMINE METABOLISM

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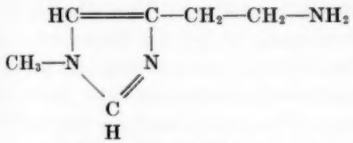
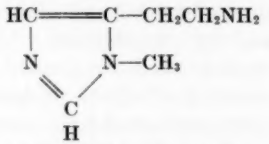
In several publications from this laboratory the existence of an unidentified major metabolic route for physiological amounts of histamine has been demonstrated (1–3). The enzyme system involved was tentatively called "histamine-metabolizing enzyme II."

We shall now present evidence that this metabolic pathway consists of two steps: (a) methylation of the imidazole ring nitrogen remote from the side chain to give 1-methyl-4(β-aminoethyl)imidazole,[1] and (b) oxidation of this methylated histamine to 1-methylimidazole-4-acetic acid. The structural formulas of the two possible ring N methylhistamines are given.



1-Methyl-4(β-aminoethyl)imidazole; (1,4-methylhistamine)



1-Methyl-5(β-aminoethyl)-imidazole; (1,5-methylhistamine)

When minute quantities of C^{14}-histamine are injected into animals, and paper chromatograms of the urine developed in butanol 80, ethanol 10, and ammonia 30, three radioactive peaks are obtained. Peak 1 results from a conjugate of imidazoleacetic acid with ribose (7), and Peak 2 from methylimidazoleacetic acid (in mouse urine Peak 2 also contains a small amount of free imidazoleacetic acid); Peak 3 contains histamine, methyl-

* Supported by a contract with the United States Atomic Energy Commission.

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[1] 1-Methyl-4(β-aminoethyl)imidazole will be referred to as 1,4-methylhistamine. This compound was generously provided by Dr. Reuben Jones of Eli Lilly and Company. Additional material was prepared by one of the authors (S. A. K.) by the method of Pyman (4). Other abbreviations to be used are ImAA-riboside for 1(3)-ribosylimidazole-4(5)-acetic acid (5,6), 1,4-methyl-ImAA for 1-methylimidazole-4-acetic acid, Marsilid for 1-isopropyl-2-isonicotinylhydrazine, and IBINH for 1-isobutyl-2-isonicotinylhydrazine.

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histamine, and a small amount of acetylhistamine. In the opinion of the authors these are the major products of histamine metabolism in the species studied (rats, mice, and guinea pigs). There is evidence, however, for the existence of unidentified minor metabolites.

Rat urine chromatograms have a very small Peak 2; hence it has been presumed that this species does not methylate histamine to a major extent. For this reason mice and guinea pigs were used in all experiments reported in this paper.

EXPERIMENTAL

Isotopic Histamine—This compound was prepared by decarboxylation of L-histidine labeled in the 2 position of the imidazole ring (8). The specific activity is 3.4×10^7 c.p.m. per mg. counted in a flow counter.

Isotope Dilution Assays—All histamine metabolites were assayed by adding an amount of carrier equivalent to 206 mg. of the corresponding picrate and crystallizing to remove impurities. In the first crystallization, water was used as the solvent and Norit as an adsorbent; in the second crystallization water-ethanol solvent (1:1) with Darco adsorbent was used and, in the third, ethanol solvent with Nuchar adsorbent. These three combinations of solvent and adsorbent were used alternately until constant radioactivity was attained. All counts were made at infinite thickness. In cases in which the amount of crystalline material was too small to permit counting at infinite thickness, it was accurately diluted and the observed count multiplied by a correction factor. Removal of isotopic impurities by recrystallizing the carrier proved to be easy in most cases. This is evident from the fact that some of the tested compounds could be added to urine containing highly radioactive compounds closely related in structure, and then recovered, free of radioactivity, by repeated crystallization. However, as a precaution some of the more difficult separations were first proved feasible; *e.g.*, non-isotopic 1,4-methylhistamine carrier to which was added a minute amount of highly radioactive histamine could be completely freed of radioactivity by this process. Activity was considered constant only if at least three successive values were unchanged.

First Evidence of Methylation of Histamine—One approach to the elucidation of the metabolic products of histamine was the injection into animals of large non-physiological quantities of histamine, followed by the isolation of radioactive substances from the urine by column chromatography. In this manner a substance was isolated from mouse urine which had microanalytical data consistent with methylimidazoleacetic acid.[2]

The next observation was the effect of large amounts of 1,4-methylhis-

[2] This material was later identified as a mixture of the two isomers of *N*-methylImAA (9).

tamine on histamine metabolism. Mice injected with 5 mg. of 1,4-methylhistamine, and then a few micrograms of C^{14}-histamine, showed a major disturbance in the pattern of chromatograms of urine. Peak 1 was unaffected, Peak 2 greatly decreased, and Peak 3 greatly increased. This observation was consistent with the hypothesis that C^{14}-histamine was being methylated, trapped by the large concentration of non-isotopic methylhistamine, and excreted before significant oxidation could occur. Isotope dilution of this urine showed the presence of 1,4-methylhistamine.

Assay of Mouse Urine—For a complete study of the possible metabolic products of histamine it was necessary to obtain 1,5-methylhistamine. This was also kindly supplied by Dr. Reuben Jones. The two isomers of methylimidazoleacetic acid had already been prepared by one of the authors (9).

Four female mice, 23 gm. average weight, were each injected subcutaneously with 17 γ of C^{14}-histamine. Urine was collected at 45 minute intervals for a total of 8 hours. A portion of the pooled urine was diluted to 10.00 ml. and assayed at zero thickness.[3] For each isotope dilution assay, 1.00 ml. of the diluted urine was used.

A description of the isotope dilution assay for each compound follows.

Histamine—The activity of the picrate remained constant from the third through the ninth crystallization. The average value during the constant period was 1380 c.p.m.

Acetylhistamine—The activity of the picrate was low after the first crystallization and dropped to a constant value from the fourth through the seventh crystallization; average value, 40 c.p.m.

1,4-Methylhistamine—The activity of the picrate remained constant from the third through the seventh crystallization; average value, 930 c.p.m.

1,5-Methylhistamine—The activity of the picrate dropped continuously and was devoid of radioactivity after nine crystallizations.

1,4-Methylimidazoleacetic Acid—The activity of the picrate remained constant from the fourth through the eighth crystallization; average value, 1610 c.p.m.

1,5-Methylimidazoleacetic Acid—The activity of the picrate was low after one crystallization, and devoid of radioactivity after the fourth.

Free Imidazoleacetic Acid—The activity of the picrate remained constant from the eighth through the eleventh crystallization; average value, 320 c.p.m.

[3] Under the conditions we routinely use for counting, the ratio of the counts per minute obtained at zero thickness to the counts per minute obtained at infinite thickness, when 206 mg. of carrier are used, is 21:1. The dilute urine had an activity of 105,000 c.p.m. per ml. Hence the sum of the activities of all the radioactive constituents, when assayed at infinite thickness, should be 105,000/21 = 5000 c.p.m.

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Conjugated Imidazoleacetic Acid—The urine was first hydrolyzed in a sealed tube at 150° for 5 hours in 1 N hydrochloric acid. The activity of the picrate remained constant from the fourth through the sixth crystallization; average value, 1050 c.p.m. for total imidazoleacetic acid. Since the value for the free form was 320 c.p.m. (see above), the value due to imidazoleacetic acid-riboside was 730 c.p.m.

The sum of the activities of all the picrates is 5010 c.p.m., the theoretical value 5000 c.p.m. The closeness of the two values is not indicative of the accuracy of the method. Errors as high as 10 per cent may often occur.

In summary, a quantitative analysis for histamine excretory products in mouse urine, expressed as per cent of the total, shows histamine 27, 1,4-methylhistamine 19, 1,4-methylimidazoleacetic acid 32, acetylhistamine 1, free imidazoleacetic acid 6, and imidazoleacetic acid-riboside 15.

TABLE I

Isotope Dilution Assay for Histamine Excretory Products in Guinea Pig Urine

Compound	No. of crystallizations during which constant activity obtained	Average c.p.m. during constant period	Per cent of total, c.p.m. per 6800
Histamine	2–5	730	11
Acetylhistamine	2–4	100	1
1,4-Methylhistamine	3–6	320	5
1,5-Methylhistamine		0	0
1,4-Methyl-ImAA	2–7	1730	26
1,5-Methyl-ImAA		0	0
ImAA-riboside		*	*

* ImAA-riboside was determined roughly by paper chromatography. This urine sample showed Peak 1 (ImAA-riboside) to contain about twice as much radioactivity as Peak 2 (1,4-methyl-ImAA). This would account for most of the remaining radioactivity. There is little, if any, free ImAA in guinea pig urine (1).

Assay of Guinea Pig Urine—Three female guinea pigs, 350 gm. average weight, were each injected subcutaneously with 17 γ of C^{14}-histamine. Urine was collected for 8 hours. The activity was 72,000 c.p.m. per ml. at zero thickness. 2 ml. were used for each isotope dilution assay. Hence the theoretical count per minute of the metabolites, assayed after addition of 206 mg. of the corresponding picrate, was $72{,}000 \times 2 \div 21 = 6800$ c.p.m. The results of the assay are shown in Table I.

Assay of Urine of Mice and Guinea Pigs Administered Inhibitor of Histamine-Metabolizing Enzyme II—Before the dual nature of "histamine-metabolizing enzyme II" was discovered, it was known that Marsilid inhibited the formation of the compound of Peak 2 in mice and guinea pigs which were given C^{14}-histamine (2). Later, during a screening of Marsilid ana-

logues for this inhibitory power, it was found that IBINH was even more potent (10). These compounds are also monoamine oxidase inhibitors (11, 12).

It was now necessary to ascertain which reaction was blocked by IBINH, the methylation reaction or the subsequent oxidation reaction. This was done both by paper chromatography and by isotope dilution assay.

When mice and guinea pigs are given aminoguanidine, diamine oxidase is inhibited, and no ImAA-riboside (Peak 1) is formed. If both aminoguanidine and IBINH are used, only one peak (Peak 3) is found in chromatograms developed in butanol 80, ethanol 10, and ammonia 30. If a chromatogram of this urine is now developed in ethanol 95, 0.1 N hydrochloric acid 5, a separation of histamine and 1,4-methylhistamine is achieved, and the presence of the latter compound is demonstrated. Methylhistamine is the faster moving of the two compounds. The compounds can be counted either directly on the paper or after elution with 0.0001 N hydrochloric acid.

The presence of methylhistamine was confirmed by isotope dilution assay. In one experiment with a group of mice given both aminoguanidine (10 γ per gm.) and IBINH (70 γ per gm.) and then 0.5 γ per gm. of C^{14}-histamine, the urine, which had no significant Peak 1 or Peak 2, assayed histamine 45 per cent, 1,4-methylhistamine 55 per cent, and 1,5-methylhistamine 0. Similarly, urine from a group of guinea pigs given both inhibitors assayed histamine 56 per cent, 1,4-methylhistamine 44 per cent, 1,5-methylhistamine 0.

The inhibitor, IBINH, thus does not block methylation of histamine; it prevents the oxidation of 1,4-methylhistamine to 1,4-methylimidazoleacetic acid.

Injection of ethionine into mice prior to C^{14}-histamine results in some suppression of Peak 2. This is presumed to result from a blocking of the methylation reaction, but it is far from complete even with high concentrations of ethionine.

DISCUSSION

The experiments described in this paper use minute amounts of injected C^{14}-histamine. They are designed to test the metabolic pathways of histamine released from a bound condition in mammalian tissues. It is this histamine which is of great importance in allergic and inflammatory conditions. There is another source of histamine, that absorbed from the intestine. The findings presented in this paper are not necessarily valid for intestinal histamine.

Both guinea pigs and mice show a remarkable specificity in methylating physiological quantities of histamine only in the ring nitrogen remote from

the side chain. Presumably the other ring nitrogen is protected from methylation by steric effects of the side chain. When mice are given large doses of histamine, however, both ring nitrogens are methylated. Karjala and Turnquest isolated a mixture of the 2 isomers of methyl-ImAA which contained about 25 per cent of the abnormal 1,5 isomer (9).

We have not attempted to demonstrate whether methylation of the side chain nitrogen occurs. As this compound would be further metabolized to give ImAA and ImAA-riboside, it was of less interest to us than the intermediate in the formation of the unidentified compound of Peak 2. Its possible occurrence will be tested in the near future.

Investigation *in vitro* of the histamine-methylating enzyme and of the oxidative enzyme is in progress. The latter may be a monoamine oxidase since it is inhibited by Marsilid and IBINH, both strong inhibitors of monoamine oxidase. However, a monoamine oxidase preparation from mouse liver which readily oxidized tyramine had no detectable effect on 1,4-methylhistamine when measured manometrically.[4] Furthermore, choline-*p*-tolyl ether bromide, which we have invariably found to inhibit the action of monoamine oxidase on several substrates in intact animals (13), had no detectable effect on the formation of methyl-ImAA in mice. It is highly improbable that the oxidative enzyme is diamine oxidase because aminoguanidine, the most powerful diamine oxidase inhibitor known, does not inhibit the formation of methyl-ImAA.

SUMMARY

A hitherto unidentified enzyme system called "histamine-metabolizing enzyme II" has been found to be a composite of a methylating enzyme and an oxidative enzyme. Physiological quantities of histamine injected into mice and guinea pigs are in part metabolized by methylation of the ring nitrogen remote from the side chain. Some of this methylhistamine, 1-methyl-4(β-aminoethyl)imidazole, is excreted unchanged in the urine. Some is oxidized to 1-methylimidazole-4-acetic acid. There is no methylation in the nitrogen adjacent to the side chain. When animals are given a powerful inhibitor of "histamine-metabolizing enzyme II," 1-isobutyl-2-isonicotinylhydrazine, methylation of histamine proceeds but the subsequent oxidation reaction is blocked. The properties of the methylating enzyme and of the oxidative enzyme are under investigation. Quantitative analyses of histamine metabolites in mouse and guinea pig urine are given. It is believed that all major histamine metabolites are now identified.

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PYRIMIDINE METABOLISM

I. ENZYMATIC PATHWAYS OF URACIL AND THYMINE DEGRADATION*

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(Received for publication, November 8, 1955)

A study of the incorporation of uracil, uridine, and uridylic acid into the ribonucleic acid of rat liver slices (1) showed the presence of enzyme systems in liver that rapidly degrade pyrimidine compounds. As a consequence of this observation, an inverse relationship between uracil incorporation into liver RNA[1] and the degradation of this pyrimidine with liberation of CO_2 was noted. The present study deals with the enzymatic pathways involved in the degradation of uracil and thymine. The work of Fink *et al.* (2, 3) indicated that the first step in the catabolism of thymine by the rat is its reduction to dihydrothymine, with the subsequent formation of β-ureidoisobutyric acid and β-aminoisobutyric acid; a similar pathway for the breakdown of uracil was suggested. The present study shows that this pathway is common to both uracil and thymine, that the initial reduction is TPNH-dependent, and that the cleavage of the corresponding β-ureido compounds is not phosphorolytic in nature as in the breakdown of citrulline (4–6).

EXPERIMENTAL

Rat livers were homogenized in 0.4 volume of 0.1 M Na phosphate buffer, pH 7.4, at 0°. The homogenate was centrifuged at 100,000 × *g* for 90 minutes at 0°, and the supernatant fluid was removed carefully to avoid contamination with the particles. The supernatant fluid was used as such or was converted to acetone-dried powder by stirring with 60 volumes of

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† The author wishes to acknowledge a fellowship grant from The Squibb Institute for Medical Research.

[1] The abbreviations used are RNA, ribonucleic acid; TCA, trichloroacetic acid; DPN and DPNH, diphosphopyridine nucleotide (oxidized and reduced forms, respectively); TPN and TPNH, triphosphopyridine nucleotide (oxidized and reduced forms, respectively); ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; IMP, inosine monophosphate; G-6-P, glucose-6-phosphate; Tris buffer, tris(hydroxymethyl)aminomethane; DHU, dihydrouracil; β-UPA, β-ureidopropionic acid; EDTA, ethylenediaminetetraacetate.

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cold (−20°) acetone and was subsequently filtered. Under these conditions 42 ml. of clear supernatant fluid were obtained from 100 gm. of rat liver and were converted to 3.6 gm. of acetone-dried powder.

Uracil-2-C^{14} was obtained enzymatically from orotic acid-2-C^{14} as described previously (1). β-Ureidopropionic acid labeled in the ureido group with C^{14} (UPA-C^{14}) was obtained by condensing β-alanine with potassium cyanate; this was subsequently heated with HCl to yield dihydrouracil-2-C^{14} (DHU-2-C^{14}) (7). Crystalline alcohol dehydrogenase of yeast was prepared according to the method of Racker (8). Glucose-6-phosphate dehydrogenase was obtained from Dr. S. Rieder and thymine-2-C^{14} (9) from Dr. A. D. Welch.

Incubation Conditions—Acetone-dried powder, 100 mg., or its equivalent of supernatant fluid, was used; the acetone-dried powder was extracted with 1.0 ml. of 0.05 M Tris buffer, pH 7.3, at 0°. To this was added 0.45 μmole of uracil-2-C^{14}, 5.0 μmoles of DHU-2-C^{14}, or 5.0 μmoles of UPA-C^{14}. The final incubation volume was 3.0 ml. Of this, 0.5 ml. was removed at zero time and added to 0.2 ml. of 10 per cent TCA, to precipitate the proteins. After centrifugation, a sample of the supernatant fluid was plated on a nickel planchet (Tracerlab), oven-dried at 80° for 4 to 16 hours, and counted. The reproducibility of this method was within ±5 per cent. Subsequent samples taken at various times of incubation were similarly treated. Within the accuracy of the method the recovery of $C^{14}O_2$ as $BaC^{14}O_3$ after enzymatic degradation of the three substrates (uracil-2-C^{14}, DHU-2-C^{14}, UPA-C^{14}) was the same as the loss of C^{14} by the plating procedure. This method was therefore used to measure the extent of the reactions.

RESULTS AND DISCUSSION

Distribution of Enzymatic Activity—According to the data of Table I, the complete enzyme system which decomposes uracil-2-C^{14} to yield $C^{14}O_2$ resides mainly in the supernatant fluid of the rat liver homogenate. The enzymatic activity of rat liver slices approximates that of the unfortified supernatant fluid. When the tissue was homogenized, a loss in enzymatic activity was found. Addition of microsomes to the supernatant fluid also resulted in an inhibition of enzymatic activity. It is therefore conceivable that, although the particulate fractions were found to be unable to degrade uracil as reported by Rutman *et al.* (10), the enzymatic systems may be present in these various fractions, but the activity cannot be exerted because of some inhibiting factor; *e.g.*, the TPNH oxidase found by Dr. B. La Du[2] to occur in microsomes. This enzyme could oxidize the TPNH which, as will be shown, is required by the enzymatic system under study.

[2] Personal communication, Dr. Bert La Du, National Heart Institute, National Institutes of Health, Bethesda, Maryland.

Cofactor Requirements—Fig. 1 shows that the enzymatic activity of the liver supernatant fluid was retained by the extracts of the acetone-dried powder. Although a 3 hour dialysis of such extracts resulted in partial

TABLE I

Degradation of Uracil-2-C^{14} to $C^{14}O_2$ by Rat Liver Fractions

The equivalent of 1 gm. of liver, wet weight, of the corresponding fractions was incubated for 30 minutes in the presence of 0.45 μmole of substrate.

Per cent of total added radioactivity					
Liver slices	Whole homogenate	Supernatant fluid	Fortified supernatant fluid*	Supernatant fluid plus fluffy layer†	Supernatant fluid and fluffy layer‡
50	22	60	98	35	25

* Contains 300 γ of TPN, 7 mg. of G-6-P, and 0.05 ml. of G-6-P dehydrogenase.
† 0.4 mg. of dry weight of fluffy layer.
‡ 0.8 mg. of dry weight of fluffy layer.

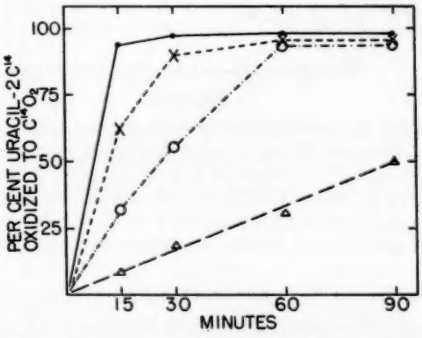

FIG. 1. Reactivation of acetone-dried powder extract with DPN and TPN after 3 hours dialysis. ○, extract of 100 mg. of acetone-dried powder of rat liver incubated with 0.45 μmole of uracil-2-C^{14}; △, same as ○ after dialysis for 3 hours against Tris buffer; ×, same as △, but with DPN (4 mg.) plus alcohol dehydrogenase and ethanol (0.1 ml.); ●, same as △, but with TPN (300 γ) plus G-6-P (7 mg.) and G-6-P dehydrogenase.

inactivation, this activity was restored completely and indeed increased by the addition of TPN plus G-6-P dehydrogenase and G-6-P as a hydrogen source; DPNH or DPN plus alcohol dehydrogenase of yeast and ethanol also was effective under these conditions. After prolonged dialysis (24 to 40 hours) against 0.05 M Tris buffer, the acetone powder extract was completely inactive (Fig. 2). Under these conditions the activity could not be restored by the addition of DPNH or DPN plus alcohol dehydrogenase

and ethanol unless TPN was present. Enzymatic activity was regained completely (Fig. 2) following the addition of TPN, G-6-P dehydrogenase, and G-6-P. This indicates the possible presence of a DPN-TPN transhydrogenase (11) which can form TPNH from TPN if DPNH is supplied. In some of the early experiments, activation apparently was obtained by the addition of ATP, ADP, AMP, or IMP to the undialyzed supernatant fluid. The cause of this activation was not pursued since it was not reproducible, and, in later experiments with dialyzed extracts of the acetone-dried powder, a requirement for these compounds was not observed.

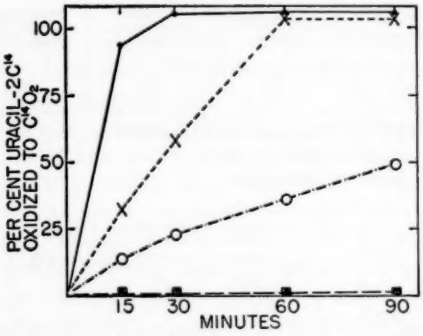

FIG. 2. Reactivation of acetone-dried powder extract with TPN after 24 to 40 hours dialysis. ×, extract of 100 mg. of acetone-dried powder of rat liver incubated with 0.45 μmole of uracil-2-C^{14}; △, same as × after dialysis for 24 to 40 hours against Tris buffer; □, same as △, but with DPN plus ethanol and the alcohol dehydrogenase of yeast; ●, same as △, but with TPN plus G-6-P and G-6-P dehydrogenase; ○, same as △, but with DPN plus ethanol, alcohol dehydrogenase, and TPN.

Formation of DHU, β-UPA, and β-Alanine—The formation of DHU in this reaction and its subsequent hydrolysis to yield β-UPA have been demonstrated by incubating uracil-2-C^{14} and acetone-dried powder extracts in the presence of 5 mg. of carrier DHU or β-UPA. These amounts of carrier are in excess of those which can be degraded by the enzyme systems under study. At the end of the incubation additional carrier (150 mg.) was added, and this was then isolated. After the second and third recrystallization, values of 23 and 24 c.p.m. per mg. and 24 and 25 c.p.m. per mg., respectively, were obtained for the DHU and β-UPA. β-UPA and β-alanine were identified by paper chromatography, the former with a solvent consisting of isopropanol, concentrated HCl, and water (85:20.5:19.5 volume per volume per volume) (12) had an R_F of 0.92, while the latter, with a solvent consisting of phenol and water (100:26 weight per volume), had an R_F of 0.60.

The DHU formed by the reduction of uracil is unstable at pH above 10. In the presence of 0.1 N NaOH it is hydrolyzed to β-UPA, which was identified by paper chromatography as previously described. This conversion was followed spectrophotometrically at 253 mμ by the disappear-

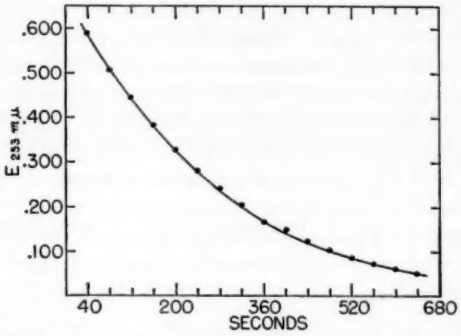

FIG. 3. Time-course of decomposition of DHU in 0.1 N NaOH. The optical density of a 5.6×10^{-4} M solution of DHU in 0.1 N NaOH was measured at 253 mμ at various time intervals.

TABLE II

Rates of Degradation of Uracil-2-C¹⁴, DHU-2-C¹⁴, and UPA-C¹⁴ to $C^{14}O_2$

Extract of 100 mg. of acetone-dried powder dialyzed for 24 hours against 0.05 M Tris buffer, incubated with the substrates in 0.05 M Tris buffer, pH 7.3.

Incubation time, min.	Per cent of total added radioactivity			
	Uracil-2-C¹⁴	Uracil-2-C¹⁴	DHU-2-C¹⁴	UPA-C¹⁴
	0.45 μmole*	0.90 μmole*	5.0 μmoles	5.0 μmoles
15	80	19	85	83
30	98	42	95	97
60		70		

* Fortified with TPN, G-6-P, and G-6-P dehydrogenase.

ance of the end-absorption characteristic of DHU. In Fig. 3 is seen the time-course of this chemical reaction.

Rates of Degradation of Uracil-2-C¹⁴, DHU-2-C¹⁴, and UPA-C¹⁴.—Incubation of uracil-2-C¹⁴, DHU-2-C¹⁴, and UPA-C¹⁴ with acetone powder extracts indicate (Table II) that, whereas DHU and β-UPA are degraded at similar rates, the rate of degradation of uracil is appreciably slower. When 5.0 μmoles of uracil-2-C¹⁴ are added, the sluggishness of the reaction does

not permit the rate to be measured with sufficient accuracy. This establishes the rate-limiting reaction as being the initial reductive step and explains the difficulty (3) encountered in the isolation of these intermediates.

Effect of Arsenate and Phosphate—The possibility that the cleavage of β-UPA to β-alanine is of the citrullinase type (4–6), involving a formation of ATP from ADP, was investigated. Acetone-dried powder extracts were dialyzed for 40 hours against three 4 liter changes of 0.05 M Tris buffer, pH 7.3, and then were incubated in the presence of ADP, ATP, phosphate, or arsenate. From Table III it may be seen that both arsenate and phosphate inhibit the reaction, whereas neither ADP nor ATP has an effect. Mg^{++} is not required, and EDTA does not inhibit the reaction. It is apparent, therefore, that in contrast to the citrullinase reaction the cleavage of β-UPA is not phosphorolytic in nature.

TABLE III

Effect of Mg^{++}, ADP, ATP, Phosphate, Arsenate, and EDTA on Degradation of UPA-C^{14} to $C^{14}O_2$

Extract of 75 mg. of acetone-dried powder dialyzed for 24 hours against 0.05 M Tris buffer, pH 7.3, and incubated with 5.0 μmoles of substrate in Tris buffer.

Time	Per cent of total added radioactivity						
	None	Mg 5×10^{-3}*	ADP, $PO_4^{=}$ 2.3×10^{-3}*	ATP 2.5×10^{-3}*	$PO_4^{=}$ 7.5×10^{-2}*	Arsenate 3.2×10^{-3}*	EDTA 0.5×10^{-2}*
min.							
15	56	53	45	46	35	30	59
30	95	94	96	96	90	80	95

* Additions (the figures indicate final molarity).

TABLE IV

Degradation of Thymine-2-C^{14} to $C^{14}O_2$

Extracts of 100 mg. of acetone-dried powder dialyzed for 24 hours against 0.05 M Tris buffer, pH 7.3, and incubated with 0.45 μmole of thymine-2-C^{14} in Tris buffer.

Incubation time	Per cent of total added radioactivity				
	Additions				
	None	DPN, ethanol alcohol dehydrogenase	TPN, G-6-P, G-6-P dehydrogenase	Same as 3rd column plus ADP, $PO_4^{=}$ 2.5×10^{-3} M	Same as 3rd column plus EDTA 0.5×10^{-2} M
min.					
15	0	0	40	45	39
30	0	0	78	76	80
60	0	0	95	98	96

Experiments with Thymine-2-C^{14}.—Because of the detailed work on the degradation of thymine by Fink *et al.* (2, 3), the present experiments were limited to establishing the requirements of the system. The enzymes involved in thymine degradation are present in the acetone-dried powders already described. No cofactors other than TPN could be shown to be required for the conversion of thymine-2-C^{14} to $C^{14}O_2$, and the reaction is not inhibited by EDTA (Table IV); TPN is essential and cannot be replaced by DPN.

These experiments have shown that an active enzyme system occurs in the rat liver capable of degrading both uracil and thymine. They have further indicated that uridine does not appear to be a *necessary* intermediate in the degradation of uracil as has been suggested (10). The liver has been shown (13, 14) not to incorporate uracil into RNA, and this has been correlated (1) to the rapid catabolism of uracil. It is interesting to note that intestinal mucosa which has been shown to incorporate uracil into RNA (15) lacks the enzyme systems involved in uracil catabolism.[3]

SUMMARY

Acetone-dried powder extracts of rat liver have been prepared which will catalyze the degradation of uracil-2-C^{14} to $C^{14}O_2$ and β-alanine. The intermediates of this reaction have been found to be dihydrouracil and β-ureidopropionic acid.

TPNH is required for the formation of dihydrouracil; the conversion of β-UPA to β-alanine appears not to be phosphorolytic in nature.

The enzymatic system which degrades thymine is present in the same fraction and has similar requirements.

The author wishes to express his appreciation to Dr. A. D. Welch and Dr. C. E. Carter for their interest in this work. Thanks are due to Miss Coryce Ozanne for her capable technical assistance.

Addendum—Papers pertinent to this topic have appeared by the following authors since this manuscript was submitted for publication: Grisolia and Wallach; and Fink, R. M., McGaughey, Cline, and Fink, K. (16).

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HYPOXANTHINE AS A COFACTOR FOR THE ENZYMATIC OXIDATION OF SULFITE*

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The oxidation of sulfite to sulfate by liver has been shown to be catalyzed by an enzyme system, of which some of the properties have been described (1–3). In continuation of these studies it was found that a partially purified enzyme preparation was inactivated by dialysis and reactivated by a thermostable factor from liver. This factor has been isolated and identified as hypoxanthine.

EXPERIMENTAL

Materials and Methods—The buffer used throughout these studies was 0.05 M potassium phosphate, pH 7.8, containing 0.01 per cent Versene Fe-III (Bersworth Chemical Company, Framingham, Massachusetts) to prevent autoxidation of sulfite. Protamine sulfate, hypoxanthine, xanthine, inosine, xanthosine, DPN,[1] ADP, ATP, coenzyme A, glutathione, flavin mono- and dinucleotides, TPN, and pyridoxal phosphate were commercial preparations. DL-α-Lipoic acid was generously provided by Dr. T. H. Jukes (Lederle Laboratories, Pearl River, New York). Dimercapto lipoate was prepared by reduction with borohydride, and a sample was also kindly provided by Dr. L. J. Reed. Inosine triphosphate was prepared from ATP by the procedure of Kleinzeller (4). Xanthine oxidase was a commercial preparation of the Worthington Biochemical Corporation, Freehold, New Jersey.

Protein concentrations were determined according to Warburg and Christian (5); Dowex 1 chromatography was conducted by the method of Cohn

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A preliminary report was presented to the American Society of Biological Chemists, San Francisco, April 17, 1955 (1).

† The data presented are taken from a dissertation submitted by Irwin Fridovich to Duke University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[1] The following abbreviations are used in the text: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; ADP, adenosine diphosphate; ATP, adenosine triphosphate; ITP, inosine triphosphate; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; and AMP, adenosine monophosphate.

and Carter (6). Carbohydrate was estimated with anthrone (7), pentose with orcinol (8), and amino acids with ninhydrin (9). Sulfhydryl groups were assayed with nitroprusside (10); organic sulfur was sought by an iodine-azide spot test (11). Phosphate was determined according to Fiske and Subbarow (12) and formaldehyde by the chromotropic acid procedure (13).

Enzyme Preparation—Unless otherwise stated, all manipulations were carried out at 0–5°. An acetone powder of dog liver was extracted by continuous stirring with 10 volumes of buffer for 2 hours. The resultant slurry was clarified by centrifugation and the supernatant fluid, designated as F-I, was rapidly brought to 60° by immersion in a water bath at 70° and immediately chilled in an ice bath. Mechanical stirring was maintained throughout. The precipitated protein was removed by centrifugation and the supernatant fluid (F-II) was adjusted to pH 5.5 by dropwise addition

TABLE I

Partial Purification of Sulfite Oxidase

Fraction No.	Protein concentration	O_2 uptake	Specific activity
	mg. per ml.	*μl. per min.*	*μl. O_2 per 100 mg. per min.*
I	59	2.2	3.7
II	28	2.6	9.3
III	17	3.2	18.8
IIIA	15	3.9	26.0

of 3.0 M sodium acetate buffer at pH 4.6. After 15 minutes in an ice bath, the mixture was centrifuged and the supernatant fluid was adjusted to pH 7.8 with 0.5 N sodium hydroxide and designated as F-III. To F-III, at pH 6.0, was added 0.1 volume of a 2 per cent solution of protamine sulfate, pH 5.5. The precipitate was removed by centrifugation and the supernatant fluid, after adjustment to pH 7.8, was designated F-IIIA. It was stored frozen with little loss of activity for as long as 6 months. The results of this purification scheme are presented in Table I.

Enzyme Assays—Manometric assays of enzyme activity were performed in standard Warburg micro respirometers at 37.5° with air as the gas phase. Alkali was omitted from the center wells since the oxidation of sulfite is not accompanied by the production of carbon dioxide. Each flask contained 1.0 ml. of enzyme and 20 μmoles of sodium sulfite in a final volume of 2.2 ml. of buffer.

Dye reduction assays of enzyme activity were performed at room temperature in Thunberg tubes which were carefully flushed with nitrogen several times before being evacuated and sealed. Each tube contained

1.0 ml. of enzyme, 0.1 μmole of methylene blue, and 20 μmoles of sodium sulfite in a final volume of 8.5 ml. of buffer. The reaction was started by tipping the sulfite into the main compartment, after which optical density changes were followed at 6500 A in a Coleman junior spectrophotometer.

Factor Assays—The dialyzable, thermostable cofactor for sulfite oxidation was assayed by its ability to restore activity to a well dialyzed preparation of dog F-IIIA in the dye reduction assay described above. The enzyme used in these routine assays was dialyzed for 48 hours against several changes of stirred, cold buffer, and was then frozen in small aliquots until

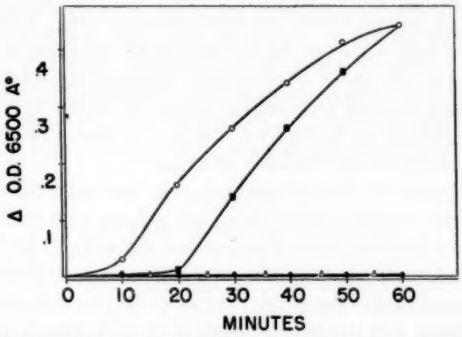

FIG. 1. The effect of dialysis of dog liver sulfite oxidase on its ability to catalyze the reduction of methylene blue. Each Thunberg tube contained 20 μmoles of sulfite, 0.1 μmole of dye, and 1.0 ml. of the indicated enzyme preparations in a final volume of 8.5 ml. buffered by 0.05 M potassium phosphate, pH 7.8, containing 0.01 per cent Versene Fe-III. ○ represents the activity of the undialyzed enzyme. The baseline represents the complete inactivity of the dialyzed enzyme (●) and of the boiled extract of the undialyzed enzyme (△), acting separately. The restoration of activity, by the boiled extract, to the dialyzed enzyme is illustrated by the middle curve (■).

needed. The amount of factor in a given solution was estimated by dilution until an aliquot (1.0 ml.) was just sufficient to allow complete decolorization of the dye. Suitable controls, containing either no enzyme or no factor, were routinely included.

Results

Dialysis of dog F-IIIA against cold buffer for 48 hours resulted in complete loss of its ability to catalyze the reduction of methylene blue by sulfite. The dialyzed enzyme was restored to full activity by a boiled extract of the undialyzed enzyme which was itself completely inactive. These findings, illustrated in Fig. 1, indicate the existence of a dialyzable, thermostable cofactor essential to the enzymatic reduction of methylene blue by sulfite.

Riboflavin, FMN, FAD, DPN, TPN, AMP, ADP, ATP, lipoic acid, oxidized glutathione, cystine, coenzyme A, pyridoxal phosphate, and thiamine pyrophosphate were found unable to restore activity to the dialyzed F-IIIA.

Properties of Factor—A boiled extract of a 10 volume aqueous homogenate of fresh calf liver proved to be a convenient source of the factor and was routinely used in the studies leading to its isolation and identification. The factor was found to be stable to 1.0 N acid or alkali at 100° for 10 minutes but was destroyed by ashing. It was not affected by nitrous acid but was destroyed by 0.1 N potassium permanganate in 10 minutes at room temperature and by 3 per cent H_2O_2 in 10 minutes at 90°. The factor was also destroyed by bromine water at room temperature. This behavior suggested that the factor contained no acid-labile phosphate or free amino groups and that it was easily oxidized and perhaps unsaturated. The factor was retained from acid solution by Dowex 50 in the hydrogen form and from alkaline solution by Dowex 1-chloride. It was, therefore, effectively cationic in acid solution and anionic in alkali.

Isolation of Factor—2 kilos of calf liver were homogenized and the homogenate poured into boiling water to a final volume of 8 liters. The slurry was boiled for 15 minutes, chilled in ice, and filtered. The filtrate was adjusted to pH 8.5 with 5.0 N KOH, and 100 ml. of saturated $BaCl_2$ were added. After addition of an equal volume of 95 per cent ethanol, the precipitate of glycogen and the barium salts of various organic phosphates was allowed to settle in the cold for 12 hours and was removed by filtration. The clear supernatant fluid was concentrated to a final volume of 1.5 liters by vacuum distillation. In 500 ml. aliquots, it was adjusted to pH 1.0 and put onto a 5 × 15 cm. column of Dowex 50, hydrogen form. The column was washed with 500 ml. of 0.1 N HCl and the factor eluted with 1 liter of 0.5 N KOH. The alkaline eluate was adjusted to pH 7.5 and the factor precipitated by addition of 0.1 volume of 1.0 M mercuric acetate. The precipitate was allowed to settle in the cold for 1 hour, collected on a Büchner funnel, and washed with 100 ml. of H_2O. The cake of precipitate was then suspended in water and decomposed with H_2S. The black residue of HgS was removed by centrifugation, the clear filtrate was freed of H_2S by forced aeration for several hours, and then made 1.0 M with respect to NH_3. It was placed on a 5 × 15 cm. column of Dowex 1-chloride, and the column was washed with 3 liters of 1.0 M NH_3, and eluted with 0.1 M NH_3, 0.01 M NH_4Cl. The eluate was collected in 250 ml. aliquots and elution of the factor was followed by its absorption at 2600 A. The peak fractions were pooled and the mercury precipitation and Dowex 1-chloride chromatography were repeated. The active eluate, freed of excess NH_3 in a vacuum desiccator over H_2SO_4, was used for the identification of the factor.

Identification of Factor As Hypoxanthine—The solution which resulted

from the isolation described above was rich in factor activity. Its absorption characteristics in acid and alkali suggested that the solution contained hypoxanthine (Table II). Assuming that the factor might contain hypoxanthine as part of its structure, the extinction coefficient of hypoxanthine at 2500 A was used as an index of the concentration of the factor in a given solution.

The factor was found to contain no carbohydrate, no pentose, no ninhydrin-reactive component, no sulfhydryl, disulfide, or organic sulfur, and no phosphate, even after vigorous acid hydrolysis. When the factor was refluxed for 8 hours in 5.0 N HCl, its characteristic ultraviolet absorption vanished and ninhydrin reactivity appeared. Chromatography on Whatman No. 1 filter paper in acetone-triethylamine-water (80:1:20)[2] suggested that the ninhydrin-reactive component might be glycine. This was established by reaction with ninhydrin, followed by distillation into bisulfite and final identification of formaldehyde in the distillate. Glycine

TABLE II

Absorption Characteristics of Factor

	Micrograms of maximal acid	Micrograms of maximal alkali	$\frac{2500}{2600}$ acid A	$\frac{2500}{2600}$ alkali A
Factor	2500	2600	1.37	0.80
Hypoxanthine	2500	2570	1.43	0.78

has been shown to be one of the products of the acid hydrolysis of hypoxanthine (14). At this point the simple identity of the factor with hypoxanthine was suspected.

Hypoxanthine completely replaced the factor in the methylene blue reduction assay. Hypoxanthine and factor were found to migrate with identical R_F on Whatman No. 1 paper in *n*-butanol saturated with water (15), *n*-butanol saturated with 0.1 N HCl (16), and in *n*-butanol-morpholine-water (3:1:4) (16).

Specificity of Factor—In the dye reduction assay, hypoxanthine could be replaced by inosine but not by ITP. Xanthine and xanthosine were approximately one-tenth as active as hypoxanthine, but this activity may have been due to hypoxanthine impurities in the commercial preparations employed. It should be emphasized that these enzyme preparations were devoid of xanthine oxidase activity and that dye reduction was dependent upon the presence of sulfite as substrate.

Hypoxanthine and Aerobic Oxidation of Sulfite—Dialysis of dog F-IIIA, while entirely eliminating its ability to catalyze sulfite oxidation with meth-

[2] Korkes, S., personal communication.

ylene blue as the electron acceptor, only slightly depressed its ability to catalyze the aerobic oxidation of sulfite. Addition of hypoxanthine effected, at most, a doubling of respiratory sulfite oxidation. If the same enzyme system is operative in both assays, since hypoxanthine is an essential cofactor to the dye reduction system, it seemed reasonable that hypoxanthine must also be involved in aerobic sulfite oxidation. Two attempts were made to determine whether dialyzed F-IIIA contained trace amounts of "bound" hypoxanthine which are adequate for maximal aerobic sulfite oxidation. Both procedures met with limited success. As illustrated in Fig. 2, passage of dialyzed F-IIIA through a 3 cm. column of charcoal re-

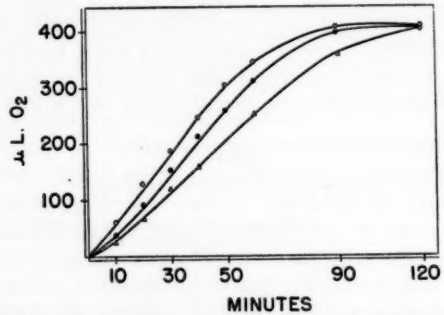

FIG. 2. The effect of charcoal treatment of dialyzed dog liver sulfite oxidase and its partial reversal by catalytic amounts of hypoxanthine. Each flask contained 40 μmoles of sulfite, 1.0 ml. of enzyme, and, where indicated, 1.0 μmole of hypoxanthine, in a final volume of 2.2 ml. buffered as in Fig. 1. ○, dialyzed F-IIIA; △, dialyzed F-IIIA passed through 3 cm. of acid-washed unground Nuchar C; ●, charcoal-treated, dialyzed F-IIIA plus 1.0 μmole of hypoxanthine.

sulted in 45 per cent inhibition which was only partially alleviated by catalytic levels of hypoxanthine. Treatment of the enzyme with purified xanthine oxidase, as shown in Fig. 3, markedly reduced the capacity for aerobic sulfite oxidation. Unfortunately, reversal of this inhibition by addition of hypoxanthine could not be attempted owing to the continued presence of xanthine oxidase. Whatever the mechanism of action of hypoxanthine in this system, it is noteworthy that the hypoxanthine added to F-IIIA respiring in the presence of sulfite could be completely recovered, as such, after respiration ceased.

Stoichiometric Requirement for Hypoxanthine—Early in these investigations it became clear that the amount of methylene blue reduced anaerobically by sulfite in assays with dialyzed dog F-IIIA was directly related to the amount of cofactor added. (For this reason the assay was peculiarly useful quantitatively and facilitated monitoring of the isolation procedure

as described above.) The stoichiometric relationship between dye reduced and hypoxanthine added is illustrated in Fig. 4. In the absence of sulfite,

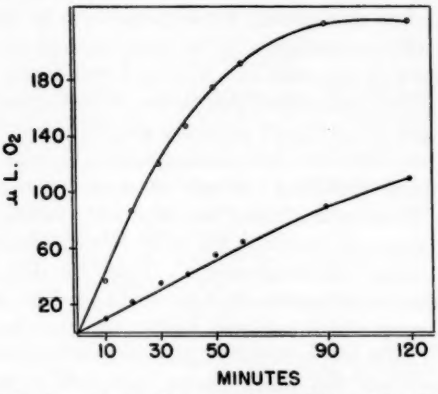

FIG. 3. The effect of xanthine oxidase on the activity of dog liver sulfite oxidase. Each flask contained 20 μmoles of sulfite and 1.0 ml. of enzyme in 2.2 ml., buffered as in Fig. 1. ○, dialyzed dog F-IIIA; ●, dialyzed dog F-IIIA preincubated for 1 hour at room temperature with 0.75 methylene blue units (17) of xanthine oxidase.

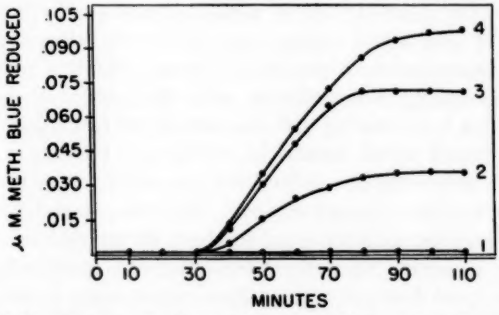

FIG. 4. Stoichiometric equivalence of hypoxanthine and methylene blue in the anaerobic oxidation of sulfite by dog liver sulfite oxidase. Each Thunberg tube contained 20 μmoles of sulfite, 0.1 μmole of methylene blue, 1.0 ml. of dialyzed dog F-IIIA, and additions of hypoxanthine, in a final volume of 8.5 ml. of buffer, as follows: Curve 1, 0.00 μmole; Curve 2, 0.03 μmole; Curve 3, 0.06 μmole; and Curve 4, 0.10 μmole.

no amount of hypoxanthine would cause reduction of the dye by the enzyme; dog F-IIIA was devoid of xanthine oxidase activity and the hypoxanthine, therefore, acted solely as a cofactor to the oxidation of sulfite.

The possibility that sulfite was somehow activating a latent xanthine oxidase activity in dog F-IIIA was checked by treating dog F-IIIA with

40 μmoles of sulfite per ml., followed by removal of the sulfite by dialysis against cold buffer. Enzyme thus treated retained its sulfite oxidase activity but remained completely inactive when assayed for xanthine oxidase.

The stoichiometric requirement for hypoxanthine in the dye reduction system is not readily compatible with the usual concept of a respiratory cofactor which serves by alternate reduction and reoxidation. As a working hypothesis, it has been postulated that in the present system hypoxanthine is first reduced to a derivative of unknown structure, and then reoxidized. However, this reoxidized form is not immediately available for reutilization under the prevailing conditions and only one turn of a respiratory cycle is possible. Since the anaerobic assay was invariably performed in the presence of a large excess of bisulfite, the most obvious explanation has appeared to lie in one of two mechanisms. Thus, the data are compatible with the possibility that the immediately reoxidized form of hypoxanthine has a carbonyl group which can bind HSO_3^- and thus be inactivated, or that the reduced form has a carbonyl group which can bind bisulfite and still permit reoxidation, but which cannot then shed its bisulfite addition compound and be reutilized. Some evidence supporting this hypothesis has been obtained.

The existence of an enzyme capable of catalyzing the desmolysis of aldehyde-bisulfite addition compounds has been reported (1) and this enzyme has been partially purified.[3] It is, therefore, noteworthy that only those preparations of liver sulfite oxidase from which this "desmolase" has been removed by treatment with protamine sulfate exhibit a requirement for external hypoxanthine stoichiometric with the amount of dye reduction. This observation is in keeping with the postulated formation of a bisulfite addition compound which cannot be reutilized. Further, a protein-free filtrate from a completed dye reduction assay could not be used to replace hypoxanthine in a second assay system. However, on standing overnight, this solution regained activity equal to about 60 per cent of the hypoxanthine initially present. Since direct formation of a bisulfite-hypoxanthine addition compound does not occur, this behavior again is compatible with the working hypotheses described above. Present studies are directed at the demonstration of a reduced form of hypoxanthine and of the nature of the final inactive form obtained in the presence of sulfite.

SUMMARY

Partial purification of the sulfite-oxidizing enzyme system of dog liver has been effected. The preparation was devoid of α-hydroxysulfonic acid desmolase activity, but catalyzed reduction of oxygen and of methylene blue. Dialysis inactivated the methylene blue reducing system; activity

[3] Fridovich, I., Eichholz, A., and Handler, P., unpublished data.

was restored by a boiled extract of active enzyme and by boiled extract of whole liver. The active principle in these extracts was identified as hypoxanthine, which appears to serve as a respiratory cofactor for the oxidation of sulfite.

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A STUDY OF THE EFFECTS OF GROWTH HORMONE ON FATTY ACID METABOLISM IN VITRO*

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As part of an investigation of regulatory mechanisms in fatty acid metabolism, a study was undertaken of the effects of the growth hormone of the pituitary gland. The well established ketogenic action of this hormone (1–6), its fat-mobilizing and respiratory quotient-lowering effects (7–12), its growth-promoting activity whereby the protein content of an animal is increased at the expense of body fat (12–14), and its apparent inhibitory action on fatty acid synthesis (15–17) all point to an involvement of this hormone in the metabolism of fatty acids. However, the biochemical sites thereof remain obscure.

The possibility that the growth hormone may exert a direct action on fatty acid catabolism in liver rests on numerous studies in which it was observed that liver preparations of animals treated prior to sacrifice with either growth hormone or anterior pituitary extracts exhibited increases in oxygen consumption or in ketone body formation in the absence or presence of added fatty acids (2, 3, 18–20). In corroboration of this idea, Bondy and Wilhelmi (4) and Tepperman and Tepperman (21) found a decreased ketogenesis in liver slices from hypophysectomized rats. However, the former investigators found that the ketogenesis of surviving liver slices of normal rats was not affected by the growth hormone, whether previously administered *in vivo* or added *in vitro*. The decrease in ketogenesis displayed by liver slices of hypophysectomized rats was attributed by Bondy and Wilhelmi (4) to reduced thyroid function, since this effect was overcome by administration of thyroxine. Shipley (22) found a 46 per cent rise in the endogenous ketone body production of liver slices from fed rats on the addition of anterior pituitary extract in an amount equivalent to 20 mg. of fresh gland. A 27 per cent increase was observed in liver slices from fasted rats. However, Campbell and Davidson (18) found no effect

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of added anterior pituitary preparations on oxygen uptake or acetoacetate production in rat liver slices, and Bondy and Wilhelmi also obtained negative results with crystalline growth hormone (4). Tepperman and Tepperman (21) observed increased ketogenesis in rat liver slices on addition of one growth hormone preparation *in vitro*, but obtained no effects with two other growth hormone preparations.

It is logical to assume that the question of the existence and the nature of a direct effect of growth hormone on fatty acid catabolism can be answered most decisively by studying its action on the catabolism of fatty acids in isolated tissue preparations. Such studies are beset by technical difficulties, however (23). Tissue slices and whole homogenates have a high endogenous oxygen consumption, and, even when an exogenous substrate increases it, there is no assurance that the difference represents the extent of oxidation of the added substrate. Ketogenesis is not a reliable criterion of fatty acid catabolism, particularly in the tissue slice in which fatty acids may also be converted to CO_2 and to amino acids. Added fatty acids, particularly those of long chain length, exert inhibitory effects on respiration which are difficult to evaluate, and in general it is our experience that ketogenesis in the presence of added fatty acids is highly variable.

Some of these difficulties can be overcome by the use of C^{14}-labeled fatty acids. By these means it is possible at least to obtain objective estimations of fatty acid oxidation and of ketogenesis by measuring the incorporation of C^{14} into respiratory CO_2 and acetoacetate, without the uncertainties involved in corrections for endogenous metabolism. In the present report, such experiments are described, slices and homogenates of rat tissues being used. These have given no indication that the purified growth hormone exerts any direct effect on the catabolism of fatty acids in rat tissues.

EXPERIMENTAL

The purified growth hormone preparations used in this study were obtained from two sources. The first one was obtained from the Frank W. Horner Company, lot No. C-24-34-D. This preparation, when given to hypophysectomized female rats in a daily dose of 10 γ over a 10 day period, brought about an average weight increase of 11 gm.[1] With female rats at a normal plateau, according to the assay procedure described by Li *et al.* (24), we found that daily injections of 0.50 mg. over an 18 day period brought about an average weight gain of 1.6 gm. per day as compared with an average gain of 0.28 gm. per day for control animals. The second preparation was obtained from the Armour Laboratories,[2] lot No. M-108.

The carboxyl-labeled fatty acids were prepared as described previously

[1] L. Mitchell of the Frank W. Horner Company, private communication.

[2] We are indebted to the Armour Laboratories for supplying us with this material.

(23) and the methyl-labeled sodium acetate and the uniformly labeled glucose (glucose-U-C^{14}) were purchased from the Isotopes Specialties Company, Inc., on allocation by the United States Atomic Energy Commission. Before use, these labeled substrates were diluted with unlabeled material to give final activities in the range of 5000 to 20,000 c.p.m., when assayed as barium carbonate according to our standard counting procedure (25). All the experiments, unless otherwise noted, were conducted with approximately 3 month-old male rats from our colony. Experiments with slices were conducted essentially as described by Weinhouse *et al.* (23), and those with homogenates as described by Wenner *et al.* (25), except that cytochrome *c* and fumarate were omitted from the incubation medium. In the small scale experiments, with 50 mg. of tissue, carrier carbonate in an amount of about 0.25 mmole was added to the contents of the center wells to facilitate recovery and assay of respiratory CO_2. Except where indicated otherwise, yields are reported in microatoms of substrate carbon converted to the product in question, calculated as described in earlier publications (25, 26).

Results

Oxidation of Fatty Acid in Liver—In experiments such as the typical ones shown in Table I, the direct addition of growth hormone to Warburg vessels in which palmitate-1-C^{14} was being oxidized by rat liver slices did not result in an increased incorporation of labeled carbon into CO_2 or acetoacetate. No differences were observed in oxygen uptake or in yields of *total* CO_2 or acetoacetate, these being similar to values reported previously in similar experiments (23). Hence, to save space, these data generally were omitted from Tables I to V. Radiochemical yields are expressed in microatoms of substrate carbon converted per hour per gm. of dry tissue, the dry weight being estimated as one-fifth of the fresh weight. These conversion values were calculated on the assumption that there is equal utilization of all fatty acid carbons (27). Radiochemical yields of acetoacetate were calculated directly from assays of carboxyl and β-carbon. With palmitate the ratio of β to COOH was 1, and with butyrate it was about 0.6; these ratios were uninfluenced by the addition of the hormone. The small differences between the supplemented and unsupplemented flasks in the utilization of palmitate carbon for CO_2 production or for ketogenesis are not regarded as significant. The conversion of butyrate carbon to CO_2 or to acetoacetate likewise was not affected significantly by the presence of growth hormone. As indicated in Experiment 4, pretreatment of the rat *in vivo* with growth hormone likewise had no effect on the oxidation of palmitate by liver slices.

The withdrawal of a citric acid cycle component via a side reaction might

result in a deficiency of oxalacetate for condensation with acetate, whereupon ketogenesis might occur as an alternate metabolic pathway. Recknagel and Potter (28) showed that the addition of ammonium chloride to rat liver slices increased ketogenesis from pyruvate, and they attributed this effect to stimulation of the amination of α-ketoglutaric acid, thereby withdrawing a potential source of oxalacetate needed for operation of the

TABLE I

Effect of Growth Hormone on Fatty Acid Oxidation in Rat Liver Slices

Each flask contained 4 to 5 gm. of fresh tissue from fed rats, and where indicated, 4 mg. of purified growth hormone (Horner preparation) suspended in 40 ml. of Ringer-phosphate solution, with oxygen in the gas phase. Incubations were carried out for 2 hours, except for Experiment 4, which was conducted for 3 hours. Palmitate concentration was 0.0005 M and butyrate 0.01 M. The values are given in microatoms of substrate carbon converted to CO_2 or to acetoacetate per gm. of dry tissue per hour. The O_2 uptakes are given in micromoles per gm. of dry tissue per hour.

Experiment No.	Substrate	O_2 uptake		Respiratory CO_2		Acetoacetate	
		Growth hormone		Growth hormone		Growth hormone	
		Absent	Present	Absent	Present	Absent	Present
		μmoles	*μmoles*	*microatoms*	*microatoms*	*microatoms*	*microatoms*
1*	Palmitate-1-C^{14}	327	313	9.5	7.9	17.3	14.0
2*	"	335	303	7.1	8.5	15.2	14.1
3	"	351	359	8.4	8.8		
	" + 0.01 M NH_4Cl	334	344	7.0	7.7		
4†	"	313	333	6.7	6.4		
5	Butyrate-1-C^{14}	375	381	69.7	79.2	234	237
	" + 0.01 M NH_4Cl	365	349	65.3	62.5	238	232

* Female rats, fasted 72 hours prior to experiment.

† Growth hormone injected *in vivo* into female rats pretreated as described for Experiment 2, Table IV.

citric acid cycle. The possibility was considered that the growth hormone might, as a consequence of its growth-promoting action, stimulate amino acid formation from citric acid cycle components, and thus promote ketogenesis. If so, it might be expected that an inhibitory effect of growth hormone on fatty acid oxidation or an enhancement of ketogenesis might be better manifested in the presence of ammonia. However, as demonstrated in Experiments 3 and 5, Table I, neither ketogenesis nor CO_2 production from palmitate or butyrate was affected by growth hormone in the presence of ammonium chloride.

Kidney—The action of growth hormone in kidney is illustrated in Table

II. This experiment is typical of a total of six thus far carried out in which the growth hormone did not enhance fatty acid oxidation. The oxidation of palmitate was depressed somewhat by the growth hormone,[3] the conversion of palmitate carbon to respiratory CO_2 having been reduced from 0.45 to 0.35 microatom. Since kidney homogenates oxidize glucose readily (25), this was included as a substrate. Glucose oxidation was also depressed by the hormone, from 3.2 to 2.1 microatoms. It was interesting to note that, even in the presence of overwhelming quantities of glucose, palmitate oxidation was as high as in the absence of added glucose and this was also true in the presence of added growth hormone. As observed previously

TABLE II

Effect of Growth Hormone on Fatty Acid and Glucose Oxidation in Rat Kidney Homogenate

Each flask contained 50 mg. of homogenized kidney from a fed rat, suspended in 1.6 ml. of isotonic KCl-phosphate solution. Each vessel contained diphosphopyridine nucleotide in a final concentration of 0.002 M and, where indicated, 0.20 mg. of growth hormone. Palmitate concentration was 0.00025 M and glucose 0.005 M. The experiments were conducted 1 hour at 38° with air in the gas phase. The values are given in microatoms of substrate carbon converted to CO_2.

Substrates	Respiratory CO_2	
	Growth hormone	
	Absent	Present
	microatoms C	*microatoms C*
Palmitate-1-C^{14}	0.45	0.35
Glucose-U-C^{14}	3.20	2.10
Palmitate-1-C^{14} + glucose	0.44	0.38
Glucose-U-C^{14} + palmitate	0.41	0.26

(29), the oxidation of glucose was greatly depressed by exogenous palmitate, but the effect was about the same in the presence or absence of added hormone. Thus, in kidney as well as in liver, growth hormone failed to influence either glucose or palmitate oxidation, and also had no effect on the oxidation of one substrate in the presence of the other.

Oxidation of Endogenous Fatty Acids—To study possible direct effects of

[3] The inhibitory effect of the growth hormone on respiration appears consistently at concentrations greater than 200 γ per ml. and is proportional to the level. This has been observed with dialyzed purified growth hormone and with whole and dialyzed pituitary extracts obtained through the kindness of C. H. Li. The inhibition was not observed with a 10-fold higher concentration of serum albumin but was observed with high concentrations of insulin. This effect, which is receiving further study, does not seem to be of physiological significance, since it is also obtained with heated preparations.

the hormone on fatty acid catabolism under somewhat more physiological conditions, we investigated the oxidation of endogenous palmitate of liver slices, labeled as previously described (29) by prior administration of palmitate-1-C^{14} to the intact rat before sacrificing. As seen in Table III, the only effect of relatively massive doses of the hormone was to inhibit both CO_2 production and ketogenesis from endogenous fatty acids by about one-third.[3] The inhibition was in the total CO_2 and acetoacetate formed and not in the specific activity, indicating that essentially all of the respiration, and not just that of the labeled fatty acids, was inhibited in the growth hormone-treated tissue.

TABLE III

Effect of Growth Hormone on Oxidation of Endogenous Fatty Acids of Rat Liver Homogenate

Each flask contained 860 mg. of homogenized liver obtained from a 24-hour fasted rat suspended in 32 ml. of isotonic KCl-phosphate solution, and, where indicated, 16 mg. of growth hormone. Each vessel also contained diphosphopyridine nucleotide in a final concentration of 0.002 M. The experiments were conducted 1 hour at 38° with air in the gas phase. The total activity values are specific activity in counts per minute times microatoms of product carbon.

Additions to medium	Respiratory CO_2			Acetoacetate		
	Amount	Specific activity	Total activity	Amount	Specific activity	Total activity
	μM	*c.p.m.*	*c.p.m.*	*μM*	*c.p.m.*	*c.p.m.*
None	121	129	15,500	13	228	11,900
Growth hormone	76	124	9,400	9	212	7,600

Acetate Metabolism in Normal Rats—In view of the lack of effect of the growth hormone on the catabolism of fatty acids *in vitro*, we turned our attention to its possible effects on lipogenesis. Inasmuch as dietary and hormonal effects on lipogenesis had been observed previously in liver slices metabolizing C^{14}-labeled acetate (17, 30–32), this system was employed in the present study. Acetate-2-C^{14} was used, and the effects of prior growth hormone administration on its incorporation into CO_2, acetoacetate, fatty acids, and cholesterol were investigated. The results of these experiments are outlined in Table IV. In general, no appreciable differences were observed in the total amounts of fatty acids and cholesterol isolated from the livers used in these experiments, and this was true also of the total yields of CO_2 and acetoacetate. The values obtained were close to those already reported in similar experiments (30), and hence are not repeated here. With the exception of the glycogen levels and the oxygen uptake values, all the data in Table IV are given in radiochemical yields, expressed as microatoms of acetate carbon converted per gm. of dry tissue per hour.

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Inasmuch as the maximal effects of growth hormone on ketogenesis (1–6) and fat mobilization (7–12) were observed within a few hours after its administration, it was felt that any effect on lipogenesis might likewise appear after such an interval. However, it was deemed advisable to test also the effect of prolonged administration.

In Experiment 1, the hormone-treated animals (3 month-old female rats from our stock colony) were given sixteen daily intraperitoneal injections of 0.5 mg. of the Horner growth hormone preparation over 19 days, during which time they exhibited the typical weight-gain response (24). After a subsequent 2 days without the hormone, growth hormone injections were resumed, and at the same time the animals were fasted for 24 hours and then refed for 24 hours with a high carbohydrate diet to insure adequate nutrition (30, 31). They were then given 15 mg. of growth hormone and sacrificed 2 to 3 hours later. Three animals each were used in the treated and control groups, and the design of these experiments followed exactly those previously reported (30, 32). Experiment 2 was conducted essentially in the same way, except that a final dose of only 5 mg. of growth hormone was given 2 to 3 hours prior to sacrifice. In this experiment, one liver from each group was allowed to oxidize palmitate-1-C^{14}, the results with these livers being presented in Table I, Experiment 4. Data on the two remaining livers of treated and control animals are represented as Experiment 2, Table IV.

In Experiments 3 to 5, normal male rats, without pretreatment with the hormone, were fasted and refed as before, and the treated groups were given 5 mg. each of growth hormone 2.5 to 4.5 hours before sacrifice, as indicated in Table IV.

It is apparent that treatment with growth hormone under the conditions employed has no appreciable effect on the glycogen-storing ability or in the respiratory capacity of liver cells. The wide variability in the capacity to incorporate acetate carbon into CO_2 or into acetoacetate makes it difficult to assess the action of the growth hormone on these processes; however, no effects are apparent which cannot be ascribed to variations among animals. The same is probably true of lipogenesis, though there is some indication of a reduced incorporation of acetate carbon into fatty acids. In the first four experiments, the incorporation of acetate into tissue fatty acids was decidedly lower in the hormone-treated animals. The extent of the difference ranged from a ratio of treated to untreated slices of 2.5:6.5 = 0.4 in Experiment 1, to 5.9:9.2 = 0.6 in Experiment 2. In Experiment 3, incorporation was decidedly lower than normal in both "treated" and "control" livers, probably a reflection of the shorter refeeding period employed in this experiment. However, in Experiment 5, the picture was reversed; *i.e.*, the incorporation of acetate carbon into tissue fatty acids was almost twice as high in the treated as in the untreated rat liver slices.

TABLE IV

Effect of Growth Hormone Injection on Metabolism of C^{14}-Methyl-Labeled Acetate in Liver Slices

Each vessel contained 400 μmoles of sodium acetate and 5.0 gm. of liver slices in 40 ml. of Ringer-phosphate saline solution. The experiments were conducted for 3 hours at 38° with oxygen in the gas phase. The values are given in microatoms of acetate carbon converted per gm. of dry tissue per hour. The glycogen determinations were conducted at the start of the experiments.

Experiment No.	Growth hormone		No. of animals and sex	Glycogen	O_2 uptake	CO_2	Acetoacetate	Fatty acids	Cholesterol
	Amount injected	Time prior to sacrifice							
	mg.	*hrs.*		*per cent*	*μmoles*	*microatoms C*	*microatoms C*	*microatoms C*	*microatoms C*
1*	15	2–3	3 F.	7.1 ± 0.5	343 ± 6	36 ± 5	23 ± 2	2.5 ± 0.9	1.8 ± 0.6
	0		3 "	5.7 ± 0.4	316 ± 10	44 ± 3	16 ± 3	6.5 ± 2.3	0.56 ± 0.04
2	5	2–3	2 "	7.2 ± 0.1	278 ± 25	16.6 ± 5.0	9.4 ± 0.8	5.9 ± 3.3	0.76 ± 0.03
	0		2 "	7.4 ± 0.4	325 ± 8	35.1 ± 3.1	16.7 ± 4	9.2 ± 1.3	0.50 ± 0.04
3	5	2.5–3.5	2 M.†	7.5 ± 0.1	322 ± 21	19.3 ± 4.7	18.4 ± 3.3	0.40 ± 0.16	0.60 ± 0.12
	0		3 "	7.1 ± 0.5	273 ± 23	16.7 ± 6.8	14.0 ± 2.1	1.00 ± 0.33	0.34 ± 0.08
4	5	3.5	1 "	9.8	300	27.2	26.0	8.00	1.17
	0		1 "	10.3	333	27.9	23.5	14.4	0.81
5	5	3.5–4.5	2 "†	6.6 ± 0.7	344 ± 17	30.8 ± 6.4	24.1 ± 0.6	9.0 ± 1.3	0.85 ± 0.03
	0		3 "	7.8 ± 1.4	306 ± 24	30.5 ± 4.7	20.3 ± 0.5	4.9 ± 1.6	0.84 ± 0.19

* All the rats were fasted for 24 hours and refed for 24 hours prior to sacrifice, except in Experiment 3, where the animals were fasted for 36 hours and refed for 12 hours. In Experiments 1 and 2, in addition to the final injection of growth hormone, animals received 0.5 mg. of growth hormone daily, Monday through Friday, for approximately 3 and 3½ weeks, respectively. Growth hormone used in Experiments 1 and 2 was prepared by the Frank W. Horner Company, Lot No. C-24-34-D; growth hormone used in the other experiments was from the Armour Laboratories, Lot No. M-108. Growth hormone was dissolved in physiological saline, pH 8.5, and was injected intraperitoneally. The control animals received saline injections.

† In both Experiments 3 and 5, one of the three livers from the hormone-injected animals was lost.

With the exception of Experiment 3, all of the values for incorporation of acetate carbon into fatty acids of "treated" liver are in the range previously observed by us for normal rat liver (30, 32). For this reason, the differences observed in these experiments between "treated" and "untreated" tissues are regarded as resulting from a secondary rather than a primary action of the hormone. Certainly the differences found are of little significance when compared with the 50-fold or greater impairment of lipogenesis occurring after a short fasting period (30, 31).

It has already been pointed out (32) that incorporation data do not necessarily give an adequate representation of fatty acid synthesis, and a procedure has been described for calculating a value which is regarded as closer to the true synthesis rate. This procedure is based on the assump-

TABLE V

*Effect of Growth Hormone Injection on Metabolism of Uniformly Labeled Glucose-C^{14} in Liver Slices**

The conditions are similar to those reported in Table IV, except for the substitution of 200 μmoles of glucose-U-C^{14} for the labeled acetate.

Growth hormone injected	Glycogen	O_2 uptake	CO_2	Acetoacetate	Fatty acids	Cholesterol
mg.	*per cent*	*μmoles*	*microatoms C*	*microatom C*	*microatom C*	*microatom C*
5	11.4	250	7.03	0.92	0.54	0.013
5	10.9	287	7.40	0.85	0.73	0.045
0	9.5	263	6.63	0.87	0.37	0.013
0	9.0	273	4.50	0.66	0.20	0.018

* Male rats fasted for 24 hours and refed for 24 hours. The growth hormone was injected intraperitoneally 3 to 4 hours prior to sacrifice (Armour lot No. M-108).

tion that the specific activity of the acetoacetate represents that of the acetyl groups undergoing conversion to fatty acids. When lipogenesis was calculated in this manner, the difference between the treated and the control groups was somewhat less than was indicated by the incorporation data. The average value for the ten "treated" livers was 15.0 microatoms as compared with an average of 17.5 microatoms of acetate carbon incorporated per gm. of dry tissue per hour for the twelve control livers.

Incorporation into Cholesterol—In the same four experiments in which the growth hormone treatment appeared to reduce the incorporation of acetate into fatty acids, there was an increased cholesterol synthesis. Only in Experiment 5 was there no difference. The average for all of the livers of the treated animals was 1.10 as compared with 0.62 microatom for the controls. The data thus suggest a possible enhancement of cholesterogenesis, but again the results are equivocal because of the variability in the data. It seems clear, however, that, if cholesterol synthesis is stimulated

line, pH 8.5, and was injected intrap
† In both Experiments 3 and 5, one of the three livers from the hormone-injected animals was lost.

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by the growth hormone, its effect is again not large compared with dietary effects on cholesterol synthesis (32).

Oxidation of and Lipogenesis from Glucose—In Table V are given the results of a similar study in which uniformly labeled glucose-C^{14} was used as the substrate. The animals used in this experiment were part of those used for Experiment 4, Table IV, and were treated exactly as described in Table IV. As was observed previously (32), oxidation of and lipogenesis from glucose in liver slices are low compared with acetate; however, again prior treatment with growth hormone did not influence appreciably the incorporation of glucose carbon into CO_2, acetoacetate, fatty acids, or cholesterol.

DISCUSSION

The results of this study provide no evidence that the growth hormone exerts a direct stimulatory effect on the enzymatic processes of fatty acid catabolism. While this work was in progress, Greenbaum and McLean (2) reported the results of a comprehensive study which led them to conclude that the growth hormone may elicit both an inhibition and a stimulation of the fatty acid oxidase system. These effects occurred, however, only within a 24 hour period after the initial administration of the hormone, and could not be correlated with changes in the respiratory quotient or with the blood and urinary ketone body levels of the intact animals. There are several technical features of these experiments which, we believe, cast considerable doubt on their validity. The fatty acid oxidase assay system employed by Greenbaum and McLean has a relatively high oxygen consumption in the absence of a fatty acid, and the oxygen consumption due to fatty acid oxidation, as well as the acetoacetate production, is obtained by difference. The assumption that the values thus obtained accurately represent the catabolism of the added fatty acid is probably not warranted. It is precisely for this reason that we have adopted the tracer technique, which, by providing an objective, independent estimation of fatty acid catabolism, avoids the uncertainties involved in correcting for endogenous metabolism.

Another uncertainty is introduced, we believe, by the employment in these experiments of succinate in a final concentration of 0.0066 M as a "sparker." This means that in each vessel there were present 20 μmoles of succinate, as compared with 3.0 μmoles of octanoate or 0.5 μmole of oleate. Inasmuch as succinate is readily oxidized in such homogenates, it seems unlikely that fatty acid oxidation can be measured from oxygen uptake data alone in the presence of such an overwhelming proportion of competing non-fatty acid carbon. Evidence for a possible inhibitory action of the growth hormone on fatty acid oxidation *in vitro* has already been men-

tioned,[3] but the physiological significance of this finding is questionable and requires further exploration.

The experiments with labeled acetate as substrate also failed to disclose any effect of growth hormone on the conversion of this metabolic intermediate to CO_2 or to acetoacetate. These findings are consistent with those of Tomkins *et al.* (33) and Baruch and Chaikoff (34), who found no change in liver slices of hypophysectomized rats in the ability to oxidize acetate.

The results of the experiments with labeled acetate suggest that the growth hormone may inhibit the synthesis of fatty acids. This finding would be in accord with several previous studies, but here also the data are not entirely consistent. Welt and Wilhelmi (15) found that the administration of growth hormone depressed the incorporation of deuterium from the body water into liver and carcass fatty acids. Brady *et al.* (16, 17) also made several observations suggestive of an inhibiting action of a pituitary principle on lipogenesis. In cat liver slices, the marked depression brought about by pancreatectomy in the incorporation of acetate carbon into fatty acids was overcome by simultaneous removal of the pituitary, and this effect could be reversed by prior administration of growth hormone. Hypophysectomy caused an increase in lipogenesis in rat liver slices, and, though the addition of growth hormone *in vitro* did not depress incorporation, it did abolish the stimulating effect of insulin addition on fatty acid synthesis *in vitro*. Confirmatory evidence of an inhibition of lipogenesis in liver slices of rats given growth hormone has been supplied by Perry and Bowen (35). However, as in our study, the differences between the control and treated animals were not large, and the percentage difference was lowered on animals fed glucose forcibly. Apparently the inhibitory effects of growth hormone on fatty acid synthesis in liver slices are relatively minor compared with the effects of short fasting periods or of diabetes (30, 31, 36, 37).

In contrast with the general impression gained by these and other studies just mentioned that the growth hormone inhibits fatty acid synthesis, Baruch and Chaikoff (34) found, surprisingly, that liver slices from hypophysectomized rats largely lost the capacity for fatty acid synthesis. It was found that cholesterol synthesis is also reduced in this condition (33), and this observation is more in line with our finding that the growth hormone somewhat accelerates cholesterogenesis in liver slices. However, it is probably incorrect to expect that the effects of hypophysectomy would necessarily be opposite to those of growth hormone administration.

In summarizing these studies in relation to the results reported by others, it appears to us that there are no grounds at present for the belief that the growth hormone exerts a direct action on the catabolism of fatty acids, either for ketogenesis or for complete oxidation to CO_2. The possibility is

recognized that conditions *in vitro* may have been unsuitable, and that possible effects were overlooked. However, attempts to demonstrate an effect of the growth hormone on fatty acid catabolism in the intact rat, to be reported separately, have likewise been unsuccessful.

Though some evidence has been brought forward to implicate this hormone in the anabolism of fatty acids, the results of this and other studies make it apparent that further work is required to reconcile conflicting and equivocal results before such an effect is established.

SUMMARY

The effect of the growth hormone on the conversion of C^{14}-labeled fatty acids to carbon dioxide or to acetoacetate was studied in rat tissue slices or homogenates under various conditions. Under no condition were these conversions accelerated by anterior pituitary growth hormone, added *in vitro*, or injected prior to sacrifice of the animals.

Liver slices from rats injected with growth hormone a few hours prior to sacrifice exhibited a somewhat lower incorporation of C^{14}-labeled acetate into fatty acids and an increased incorporation into cholesterol. These effects, however, were neither marked nor consistent.

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METABOLIC STUDIES ON HEART MITOCHONDRIA*

I. THE OPERATION OF THE NORMAL TRICARBOXYLIC ACID CYCLE IN THE OXIDATION OF PYRUVATE

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Relatively few investigations have been made on the oxidation of pyruvate by heart mitochondria. Guinea pig heart mitochondria (1) and rabbit heart cyclophorase preparations (2, 3) have been found to oxidize pyruvate, but in no case has a detailed investigation been made of the mechanisms involved and the requirements for the operation of the complete tricarboxylic acid cycle. On the other hand, the oxidation of certain intermediates of the cycle by heart mitochondria, or sarcosomes, has been studied in some detail (4–11). It was considered important, therefore, to develop techniques for isolating heart mitochondria in which the complete cycle operated as an intact unit when supplied with pyruvate, and to study the mechanisms by means of which pyruvate is oxidized as a basis for investigations of the disturbances induced in the cycle by various enzyme inhibitors.

EXPERIMENTAL

The method developed to obtain a mitochondrial preparation that was stable and highly active in the complete oxidation of pyruvate was the outcome of numerous tests with isolation media and centrifugation procedures. In this method the same medium was used for homogenization, centrifugation, and suspension of the mitochondria; it contained KCl (0.121 M), potassium phosphate buffer (0.02 M, pH 7.4), and ATP[1] (0.5 mM). The preparation was carried out at 0–2°. Four rat hearts, average total weight 3.5 gm., were minced and homogenized in 15 ml. of medium for 4 minutes. After dilution to a 10 per cent (weight per volume) suspension, a low speed centrifugation (600 × *g* for 10 minutes) was employed to remove unbroken cells, blood cells, nuclei, and fibrous material. The

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[1] The following abbreviations are used: ATP, adenosine triphosphate; AMP, adenosine monophosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; DPT, diphosphothiamine; CoA, coenzyme A.

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supernatant fluid was diluted with 100 ml. of medium, this large volume being used to reduce the endogenous respiration to a low level, and was centrifuged at 10,000 × *g* for 10 minutes. The residue was suspended in 12 ml. of medium by homogenization for 30 seconds, and 1 ml. of this mitochondrial suspension was used in each vessel. Examined by high power phase contrast microscopy, the final residue appeared as homogeneous ellipsoidal particles that stained with Janus green B.

Conventional Warburg manometers with 15 ml. vessels were used for determination of gas exchange. The reaction medium contained the following: KCl (0.121 M), potassium phosphate buffer (0.02 M, pH 6.8), cytochrome *c* (0.01 mM), $MgCl_2$ (5 mM), ATP (0.5 mM), and AMP (1 mM). Addition of the mitochondrial suspension brought the pH to 6.9 and the final volume to 3.0 ml. All substrate solutions were adjusted to pH 6.9 before addition. The center well contained 0.2 ml. of 30 per cent (weight per volume) KOH. The gas phase was air, the equilibration period 10 minutes, and the temperature of the bath 27°. In certain experiments a 1 per cent CO_2 atmosphere was produced by the method of Pardee (12).

Citrate was determined by the method of Ettinger, Goldbaum, and Smith (13), pyruvate and α-ketoglutarate by that of Friedemann and Haugen (14), and nitrogen by the method of Koch and McMeekin (15). Protein units were determined spectrophotometrically according to a method described by Harman and Feigelson (4). 1 protein unit was found to represent 0.433 mg. of N per ml. The Q_{O_2} values are expressed as microliters of O_2 per mg. of N per hour and calculated for the initial 10 minutes (Q_{O_2} 10 minutes) and the total first 60 minutes (Q_{O_2} 60 minutes).

Sodium succinate was recrystallized from warm aqueous solution by addition of ethanol according to Potter and Schneider (16). L-Malic acid was recrystallized according to a method of Ochoa[2] by dissolving in a minimal amount of boiling isobutylmethylketone, adding activated charcoal, and filtering the hot solution, to which chloroform was added to 10 per cent (volume per volume); on cooling, the malic acid crystallized. Fumaric acid was recrystallized from a hot aqueous solution. α-Ketoglutaric acid was dissolved in warm ethyl acetate and recrystallized by the addition of petroleum ether. A solution of potassium oxalosuccinate was prepared immediately before use from barium oxalosuccinate (70 per cent pure, supplied by Dr. Ochoa) according to the method of Ochoa (17). Other substrates were used as obtained commercially. The sodium pyruvate used did not contain the cycle inhibitor commonly present (18).

Results

Activity of Cycle in Rat Heart Mitochondria—The average Q_{O_2} values for the oxidation of various intermediates of the cycle and of several sub-

[2] Personal communication.

stances which can be incorporated into the cycle are presented in Table I. The preparation showed a negligible endogenous oxygen uptake. Since the oxidation of those substrates which enter the cycle as acetyl CoA requires the presence of an intermediate capable of giving rise to oxalacetate, malate (5 mM) was added routinely. Addition of DPN (0.2 mM) increased the rates of oxidation of malate and fumarate (2-fold) and TPN (0.8 mM) increased the rates of oxidation of isocitrate (20-fold), *cis*-aconitate (4-fold), and citrate (1.3-fold). Reduction of the pH to 6.3 and 5.7 brought about only a depression of the rate of citrate oxidation (82 and 66 per cent, respectively), indicating that the low oxidation rate was not due to inability to penetrate into the mitochondria.

TABLE I

Activity of Rat Heart Mitochondria

All the substrates are given at 5 mM. The Q_{O_2} for pyruvate plus malate, estimated simultaneously with every substrate, is given as the control. The medium contained KCl (0.121 M), potassium phosphate buffer (0.02 M, pH 6.8), cytochrome *c* (0.01 mM), $MgCl_2$ (5 mM), ATP (0.5 mM), and AMP (1 mM). The Q_{O_2} values are averages of five to ten experiments for each substrate.

		Q_{O_2} 10 min.	Q_{O_2} 60 min.	Control	
				Q_{O_2} 10 min.	Q_{O_2} 60 min.
Substrate	No substrate	25	8	675	606
	Pyruvate	24	7	675	606
	Citrate	83	62	781	726
	cis-Aconitate	104	83	926	803
	Isocitrate	22	114	950	834
	Oxalosuccinate	343	195	825	719
	α-Ketoglutarate	411	418	875	778
	Succinate	399	265	706	602
	Fumarate	89	75	770	676
	Malate	121	116	834	744
	Oxalacetate	361	314	831	734
Substrate + malate	Pyruvate	862	745		
	Glutamate	522	466	882	780
	β-Hydroxybutyrate	251	248	955	845
	Octanoate	126	63	810	716
	Acetate	190	145	810	716

Oxidation of Pyruvate—Plaut and Plaut (1) reported that guinea pig heart mitochondria oxidized pyruvate alone at a Q_{O_2} of 50, which was increased to 300 by the addition of malate. The mitochondrial preparations used in the present study were found to be incapable of oxidizing pyruvate alone. However, when malate was present, pyruvate was oxidized vigorously with a Q_{O_2} of 862 (thirty-nine experiments). When malate was 5

mM, the concentration of pyruvate with the highest initial oxidation rate was between 3 and 5 mM. As seen in Fig. 1, concentrations of pyruvate on either side of this range gave reduced Q_{O_2} values. The ability of the various cycle intermediates to promote pyruvate oxidation is presented

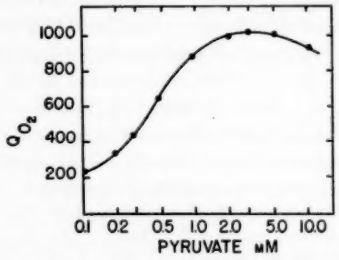

FIG. 1. Rates of oxygen uptake of heart mitochondria at various concentrations of pyruvate. Medium and conditions as in Table I. Malate at 5 mM; Q_{O_2} values are for the initial 10 minutes.

TABLE II

Oxidation of Pyruvate in Presence of Various Cycle Intermediates

All substrates are given at 5 mM. The Q_{O_2} 10 minutes for the pyruvate plus cycle intermediate is given, and also its percentage of the control, which is the Q_{O_2} 10 minutes for pyruvate plus malate. The medium is as in Table I. The Q_{O_2} values are averages of five to ten experiments.

Cycle intermediate	Test Q_{O_2}	Control Q_{O_2}	Per cent of control
	Pyruvate + intermediate	Pyruvate + malate	
Citrate	639	980	65.1
cis-Aconitate	757	1030	73.5
Isocitrate	641	980	65.4
Oxalosuccinate	713	768	92.7
α-Ketoglutarate	845	919	92.1
Succinate	1126	810	139.0
Fumarate	833	782	106.5
Malate	915	915	100.0

in Table II. Although addition of DPN increased oxidation of malate, it had no effect on the oxidation of pyruvate in the presence of malate. However, addition of TPN increased the oxygen uptake when pyruvate was being oxidized in the presence of citrate, *cis*-aconitate, or isocitrate to levels 93 to 96 per cent of the controls. It can be seen that each member of the cycle was capable of initiating and maintaining the oxidation of pyruvate.

The oxidation of pyruvate, after an initial small decrease, proceeded at a constant rate until the pyruvate was used up, at which point the oxygen uptake dropped rapidly; if more pyruvate was tipped in before this fall, a constant oxidation rate was observed up to 8 hours. That the cycle was self-perpetuating in these mitochondria, *i.e.* capable of continuously regenerating oxalacetate to condense with acetyl CoA, was shown by re-

TABLE III

Relation between Oxygen Uptake and Pyruvate Utilization

The medium is as in Table I; malate at 5 mM.

Time	Pyruvate present	Pyruvate utilized	O_2 utilized	Oxygen / Pyruvate
min.	*μmoles*	*μmoles*	*μmoles*	
0	12.64	0	0	
30	9.52	3.12	8.47	5.42
60	6.46	6.18	16.61	5.36
90	3.52	9.12	24.11	5.28

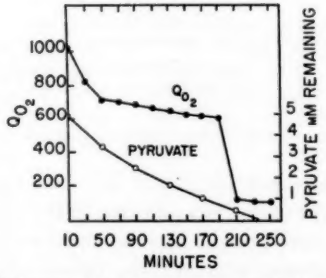

FIG. 2. Correlation between the rate of oxygen uptake and pyruvate utilization. Medium and conditions as in Table I; initial pyruvate concentration 5 mM, and malate at 5 mM. ●, oxygen uptake; ○, pyruvate concentration.

duction of the malate concentration from 5 to 0.5 mM without appreciably altering the Q_{O_2} or the total amount of oxygen consumed over a period of 4 hours. Pyruvate was completely oxidized to CO_2 and water as demonstrated in Table III, where the ratio of oxygen consumed to the pyruvate utilized is found to be somewhat higher than the theoretical ratio of 5. (The higher values were probably due to independent oxidation of malate, which was in excess of that required to initiate pyruvate oxidation.) The correlation between the oxygen uptake and the disappearance of pyruvate is illustrated in Fig. 2, from which it can be seen that failure of oxidation was rapid when the concentration of pyruvate fell below 0.5 mM, thus

agreeing well with the data presented in Fig. 1, in which the Q_{O_2} was low at pyruvate concentrations below 1 mM.

The initial decrease in the oxygen uptake over the first 30 to 40 minutes to a relatively steady level suggested that some rate-limiting mechanism had arisen to decrease the incorporation of pyruvate into the cycle. Since the tricarboxylic acids were oxidized relatively slowly, it was considered that the rate-limiting step in the steady state might be in this region of the cycle. Simultaneous determinations of pyruvate and citrate showed that during pyruvate oxidation small quantities of citrate did accumulate, as indicated in Table IV, this accumulation reaching a maximum at around 40 minutes. Presumably some *cis*-aconitate and isocitrate would also be present in equilibrium with the citrate.

The Q_{O_2} for pyruvate plus succinate was greater than that for pyruvate plus α-ketoglutarate (Table II), and yet α-ketoglutarate alone was oxidized

TABLE IV

Accumulation of Citrate during Oxidation of Pyruvate

The medium is as in Table I; malate at 5 mM.

Time	Pyruvate utilized	Citrate formed
min.	*μmoles*	*μmoles*
20	0.99	0.102
40	2.41	0.320
60	5.35	0.311

more rapidly than was succinate (Table I). The rate of oxygen uptake with succinate alone was essentially additive to the rate owing to pyruvate oxidation when both were present together, whereas, when pyruvate and α-ketoglutarate were present together, the rate of oxygen uptake was actually less than that for the pyruvate control. Competition between the pyruvic and α-ketoglutaric oxidases was considered as a possible rate-limiting mechanism. If no competition existed, the Q_{O_2}, when pyruvate and α-ketoglutarate were oxidized simultaneously, should equal the sum of the Q_{O_2} values for each substrate when oxidized separately. Table V presents data which indicate that summation does not occur and that competition is likely. Malate at 5 mM was shown to have no effect on the oxidation of α-ketoglutarate and hence played no direct rôle in this competition. It can be seen that the higher the α-ketoglutarate concentration, the greater the deviation from the calculated summation.

At the high level of malate routinely used (5 mM), formation of oxalacetate would not constitute a rate-limiting step, but at low concentrations of malate the limited quantities of oxalacetate formed, together with other

rate-limiting steps that might occur within the cycle, could cause a slowing of entry of pyruvate into the cycle, as demonstrated in Table VI. When the concentration of malate was less than 0.5 mM, some steps between malate and the entry of pyruvate into the cycle seemed to be limiting the oxidation of pyruvate.

TABLE V

Simultaneous Oxidation of Pyruvate and α-Ketoglutarate

The medium is as in Table I; malate at 5 mM.

Pyruvate (1)	Q_{O_2} (2)	α-Ketoglutarate (3)	Q_{O_2} (4)	Sum of Q_{O_2} of (2) + (4)	Q_{O_2} (pyruvate + α-ketoglutarate) (5)	Deficit, (2) + (4) − (5)
mM		mM				
1	940	1	297	1237	1000	237
1	940	5	411	1351	985	366
1	940	10	517	1457	1033	424
1	940	20	565	1505	973	532
5	951	5	411	1362	1013	349
5	951	20	565	1516	977	539

TABLE VI

Effect of Malate Concentration upon Initial Pyruvate Oxidation

The medium is as in Table I; pyruvate at 5 mM.

Malate	Q_{O_2} 10 min.
mM	
10	930
5	895
1	860
0.5	846
0.2	767
0.05	457

Requirements for Maximal Pyruvate Oxidation—The maximal rate of pyruvate oxidation was obtained when magnesium, phosphate, cytochrome *c*, and AMP were present. Pyruvate was not oxidized in the absence of added magnesium or adenine nucleotides; concentrations of 0.5 mM magnesium and 1 mM AMP gave maximal stimulation, higher concentrations either depressing the rate (AMP) or having no effect (Mg^{++}). This is in general agreement with the results on rabbit heart cyclophorase preparations (2). ATP (0.5 mM) would only partially replace AMP, allowing pyruvate oxidation to proceed at a rate about 60 per cent of the maximum. In the complete absence of added cytochrome *c*, pyruvate was oxidized at

50 per cent of the maximal rate; a concentration of 0.01 mM gave a maximal effect. A rate of pyruvate oxidation also about 50 per cent of the maximal was obtained when no phosphate was added; the concentration which resulted in maximal oxidation was found to be 10 mM, above which a slight depression was observed. The addition of the following was found to have no accelerating effect on pyruvate oxidation initiated by malate: DPT (2 mM), CoA (0.75 mM), DPN (0.2 mM), TPN (0.05 to 1 mM), Mn^{++} (1 mM), Co^{++} (0.2 to 3 mM), glutathione (1 mM), nicotinamide (1 mM), or hexokinase and glucose. A range of pH from 5.2 to 8.0 was examined, and it was found that the maximal rate of pyruvate oxidation occurred at 7.6. However, a pH of 6.9 was used throughout the investigation since at this pH the rate was only 10 per cent below the maximal, and it was

TABLE VII

Oxidation of Pyruvate in 1 Per Cent CO_2 Atmosphere

The medium is as in Table I, except that the gas phase was 1 per cent (volume per volume) CO_2; pyruvate and malate at 5 mM.

Substrates	Q_{O_2} 10 min.	
	Air	1% CO_2
None	0	0
Pyruvate	0	111
" + malate	805	820

felt that this approached intracellular conditions more closely than higher pH values.

Oxidation of Pyruvate in Absence of Cycle Intermediates—Kidney mitochondrial preparations are able to oxidize pyruvate in the absence of added cycle intermediates when bicarbonate is present (19, 20), but guinea pig heart mitochondria were reported to be unable to oxidize pyruvate under these conditions (1). Rat heart mitochondria were found to oxidize pyruvate to a limited extent in the presence of 20 mM bicarbonate (Q_{O_2} = 147 with bicarbonate and 880 with malate). Table VII shows that pyruvate could also be oxidized in the absence of added cycle intermediates when the gas phase was 1 per cent CO_2. These results imply the presence of an enzyme catalyzing CO_2 fixation.

The oxidative decarboxylation of pyruvate to acetyl CoA was demonstrated in the absence of cycle intermediates by addition of 2.5 μmoles of CoA from the side arm after the initial reading. There ensued an immediate burst of oxygen uptake (Q_{O_2} of 246) which was not maintained. In the absence of an acetyl acceptor to regenerate CoA, pyruvate uptake

ceased when the available CoA was exhausted. This implied the absence of an active acetyl CoA deacylase in rat heart mitochondria and their inability to form acetoacetate from acetyl CoA as has been demonstrated in liver mitochondria (21). Arsenate could act to a limited extent as an acetyl acceptor; $Q_{O_2} = 5$ for pyruvate alone, 81 for pyruvate plus arsenate (5 mM), and 755 for pyruvate plus malate. Arsenate (0.2 to 10 mM) had no effect on the oxidation of pyruvate in the presence of malate.

DISCUSSION

Rat heart mitochondria, supplemented with magnesium, phosphate, cytochrome *c*, and AMP, oxidize pyruvate, in the presence of a cycle intermediate, at a more rapid rate than any other substrate tested and appear capable of directing a major portion of the pyruvate through the classical stages of the cycle to complete oxidation. In the absence of a cycle intermediate to provide an acetyl acceptor, pyruvate was unable to enter the cycle, but could undergo the one-step oxidative decarboxylation to acetyl CoA until the available CoA was depleted; neither deacylation of acetyl CoA nor formation of acetoacetate from it occurs.

The sustained rate of pyruvate oxidation is presumably regulated by rate-limiting factors which come into operation after the initial entry of pyruvate into the cycle. Evidence was presented that such rate-limiting steps may exist at the tricarboxylic acid and α-ketoglutarate stages. The demonstrated accumulation of citrate during pyruvate oxidation could indicate that the aconitase reaction under these conditions cannot operate as rapidly as the condensation of pyruvate into the cycle. Accumulation of citrate during pyruvate oxidation has also been observed in dialyzed brain homogenates (22). It has been observed by Krebs and Holzach (23) that in the reaction citrate → isocitrate catalyzed by pig heart aconitase, *cis*-aconitate accumulated before isocitrate began to be formed, and this might account for the mitochondrial accumulation of citrate initially when it is supplied at a rapid rate, since the equilibrium citrate ⇄ *cis*-aconitate is actually in favor of citrate. Whether the α-ketoglutaric oxidase presents a rate-limiting reaction or not would probably depend on the concentrations of pyruvate and α-ketoglutarate present, since competition between them was observed. It is suggested that cofactors shared by the pyruvic and α-ketoglutaric oxidases might provide the basis for this competition; if so, these two oxidases must be more interdependent than is commonly assumed. Similar competition between pyruvate and α-ketoglutarate has been described in other preparations (24–26). Accumulation of α-ketoglutarate in rat heart mitochondria has been demonstrated during early stages of pyruvate oxidation (27); as the pyruvate disappeared, the α-ketoglutarate oxidation presumably increased so that

complete oxidation of pyruvate was eventually achieved. If pyruvate were at a low concentration, such as might occur in the intact cell, this competition would be less effective and this step might not then be rate-limiting. A third possible rate-limiting step in the cellular operation of the cycle might be the condensation of pyruvate into the cycle if the formation of oxalacetate were low or if an active oxalacetic decarboxylase were removing part of it as formed, since it was shown that, when malate concentration was very low, pyruvate oxidation was slower. Hence, the overall rate at which pyruvate is oxidized is governed by a balance between several factors involving both the entrance into the cycle and rate-limiting steps within the cycle.

SUMMARY

The oxidation of pyruvate by a highly active preparation of rat heart mitochondria has been investigated, and the requirements for a maximal oxidation rate were determined. In the absence of a cycle intermediate to provide oxalacetate, pyruvate is not utilized via the cycle, although acetyl CoA is formed until available CoA is depleted; no regeneration of CoA occurs unless an acetyl acceptor is added. Fixation of CO_2 can occur and initiate pyruvate oxidation to a limited extent. Three possible rate-limiting factors in the operation of the cycle are discussed and evidence for their importance under certain conditions is presented.

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METABOLIC STUDIES ON HEART MITOCHONDRIA*

II. THE INHIBITORY ACTION OF PARAPYRUVATE ON THE TRICARBOXYLIC ACID CYCLE

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Malonate and fluoroacetate, or its metabolic product fluorocitrate, are the only substances available as relatively specific inhibitors of the tricarboxylic acid cycle. Development of additional potent, specific, and permeant inhibitors of the cycle would provide useful tools for the study of the relations of the cycle to cell function. This report is concerned with a new and potent cycle inhibitor which is specific for the α-ketoglutaric oxidase. An impurity which markedly modified the operation of the cycle in rat heart mitochondria was found in various samples of pyruvic acid and sodium pyruvate and tentatively identified as the lactone of the dimer of pyruvic acid (1). However, it has now been demonstrated that the true inhibitor is not the lactone itself and that the inhibition of the α-ketoglutaric oxidase is not a simple competition with substrate, as the early work had indicated.

EXPERIMENTAL

The rat heart mitochondrial preparation and the manometric and analytical procedures were as previously described (2). α-Ketoglutaric oxidase was prepared from pig heart (3) and assayed by determination of the rate of DPN[1] reduction (4). Isocitric dehydrogenase was prepared from an acetone powder of rat heart mitochondria (5) and assayed by determination of the rate of TPN reduction (6). Oxalosuccinic decarboxylase was prepared from an acetone powder of pig heart (7) and assayed manometrically (8). The lactone of the dimer of pyruvic acid and barium parapyruvate were prepared by the method of de Jong (9). *l*-Citramalic acid, isolated from apple peel, was kindly supplied by Dr. A. C. Hulme, Department of Scientific and Industrial Research, Food Investigation, Ditton Laboratory, Maidstone, England.

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[1] The following abbreviations are used in this paper: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; CoA, coenzyme A.

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Results

The presence of an impurity in certain preparations of sodium pyruvate was shown to modify the behavior of rat heart mitochondria towards pyruvate thus: (*a*) the cycle was no longer self-perpetuating, and for the utilization of pyruvate an equimolar amount of malate, or related cycle

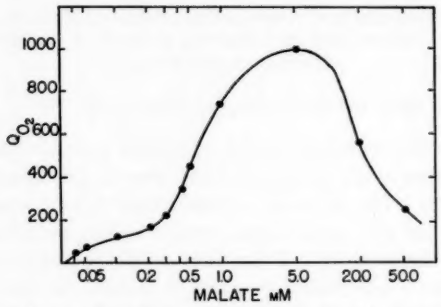


FIG. 1. Rate of pyruvate oxidation in the presence of impurity and various concentrations of malate; medium and conditions as in Table I.

TABLE I

Conversion of Pyruvate into α-Ketoglutarate during Cycle Block Caused by Pyruvate Impurity

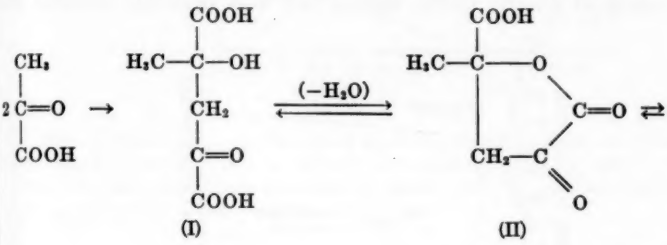
The initial concentrations of pyruvate and malate were 5 mM; medium as in Table I of Paper II. Zero time was at the first reading and after 10 minutes equilibration.

Time	Pyruvate	α-Ketoglutarate	Pyruvate + α-ketoglutarate	α-Ketoglutarate utilized	Pyruvate utilized	O_2 utilized	Oxygen / Pyruvate
min.	*μmoles*	*μmoles*	*μmoles*	*μmoles*	*μmoles*	*μmoles*	
0	12.31	0	12.31	0	0	0	
20	10.28	1.66	11.94	0.37	2.03	2.67	2.63
30	9.26	2.56	11.82	0.49	3.05	3.96	2.60
100	5.59	5.68	11.27	1.04	6.72	12.76	3.79
130	3.19	7.67	10.86	1.45	9.12	17.03	3.73

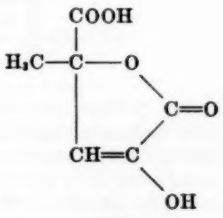
intermediate, was required, catalytic amounts of malate initiating only an insignificant pyruvate oxidation (Fig. 1); (*b*) the tricarboxylic acids, citrate, *cis*-aconitate, and isocitrate, were no longer capable of promoting pyruvate oxidation; (*c*) the oxygen-pyruvate ratio fell from the theoretical value of 5 for complete oxidation to values around 3, as shown in Table I; and (*d*) α-ketoglutarate accumulated in amounts almost stoichiometric with the amount of pyruvate utilized, as demonstrated in Table I. The accumulating α-ketoglutarate was determined simultaneously with pyruvate as

the 2,4-dinitrophenylhydrazone by making use of the different extinction coefficients of the two hydrazones at wave-lengths of 400 mμ and 540 mμ. The spectral absorption curve of the dinitrophenylhydrazone of the accumulated α-ketoglutarate corresponded closely to that of the similar derivative of pure α-ketoglutarate.

Nature of Inhibitor—Pyruvic acid by an aldol condensation can form a dimer, parapyruvic acid (γ-methyl-γ-hydroxy-α-ketoglutaric acid (I)), and this dimer can lose water to form the cyclic α-keto-γ-valerolactone-γ-carboxylic acid (II), which could exist in an enolic form (III). Titration of



(I) (II)



(III)

the lactone with KOH demonstrated that 2 equivalents of base were required for complete dissociation with $pK_1 = 2.35$ and $pK_2 = 6.95$, the first dissociation constant referring to the free carboxyl group and the second to the enolic hydroxyl (1). It has been shown that 1,2-diketo lactones usually occur almost entirely in the enolic form (10).

It was noted that the ultraviolet absorption spectrum of the lactone in aqueous solution changed with time, and it was considered that this change might be correlated with opening of the lactone ring. Such a ring opening should be detected by titration. It was found that, when the lactone stood at neutral pH, a new pK at 3.95 appeared, corresponding to a second free carboxyl group and paralleling the disappearance of the pK at 6.95. Determination of the relative amounts of the substances with the two pK_2 values at various times up to 24 hours showed that the amount of lactone became progressively less, while the amount of dicarboxylic acid became correspondingly greater, as seen in Fig. 2. It appeared that an equilibrium

was approached when the ratio of parapyruvate to lactone was 3.2:1. The opening of the lactone ring was also demonstrated in acid and alkaline solutions, the lactone being more stable under acid conditions.

The question arose as to which of the compounds was the cycle inhibitor. Samples of the lactone were allowed to stand at neutral pH for varying times and tested for inhibition of the ability of isocitrate to initiate pyruvate oxidation. As illustrated in Fig. 2, the inhibition increased with increasing concentration of parapyruvate in the solution, indicating that parapyruvate rather than the lactone was the true inhibitor. Pure parapyruvate, obtained directly as the barium salt from pyruvate, showed the

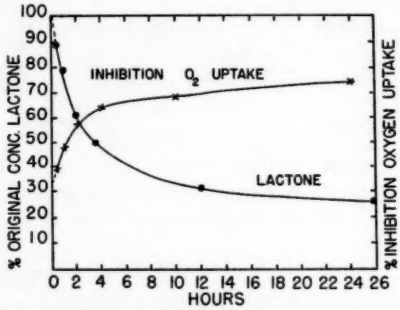


FIG. 2. Correlation of the opening of the lactone ring (formation of parapyruvate) with increasing inhibition of the ability of isocitrate to promote pyruvate oxidation; medium and conditions as in Table I. Solutions of the lactone (1 mM) allowed to stand at pH 7 for periods indicated. ●, concentration of lactone remaining (determined by titration) and ×, per cent inhibition of Q_{O_2} for pyruvate oxidation in the presence of isocitrate (5 mM).

same activity as parapyruvate obtained from the lactone. When parapyruvate was added with pure pyruvate to the mitochondrial suspension, all the phenomena described with the impure pyruvate were obtained; the cycle was no longer self-perpetuating (Fig. 3), and citrate and isocitrate were unable to initiate pyruvate oxidation, whereas the activities of succinate and malate remained unimpaired (Table II), and α-ketoglutarate accumulated during the oxidation of pyruvate. The effect of various concentrations of parapyruvate on the ability of citrate to promote pyruvate oxidation is also presented in Table II. The extinction coefficient ratios at different wave-lengths for the dinitrophenylhydrazones of the accumulated keto acid (in the presence of parapyruvate) and of pure α-ketoglutarate were quite similar; the ratio at 400:460 mμ was 1.35 for both substances, and at 420:540 mμ it was 2.16 for α-ketoglutarate and 2.15 for the accumulated keto acid. The spectral absorption curves for the two derivatives

were essentially identical. It was therefore concluded that this keto acid is identical with α-ketoglutarate.

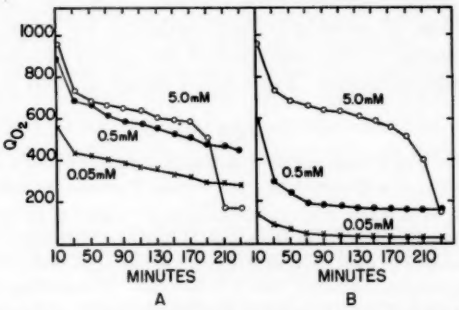


FIG. 3. Effect of parapyruvate on time-course of pyruvate oxidation; medium and conditions as in Table I. Malate concentration: ○, 5 mM; ●, 0.5 mM; ×, 0.05 mM. *A*, oxidation of pure pyruvate; *B*, oxidation of pure pyruvate in the presence of parapyruvate (1 mM). Pyruvate at 5 mM.

TABLE II

Inhibition by Parapyruvate of Ability of Cycle Intermediates to Promote Pyruvate Oxidation

Pyruvate and cycle intermediates are 5 mM; medium as in Table I.

Cycle intermediate	Parapyruvate	Per cent inhibition
	mM	
Citrate	0.0003	4
"	0.001	10
"	0.003	19
"	0.01	26
"	0.05	43
"	0.1	64
"	0.2	80
"	1	91
"	5	100
Isocitrate	1	80
α-Ketoglutarate	1	6
Succinate	1	0
Malate	1	0

Site of Inhibition—The results were interpreted to mean that parapyruvate blocked some reaction between isocitrate and succinate in the cycle. Parapyruvate in concentrations up to 5 mM did not inhibit either the isocitric dehydrogenase from rat heart mitochondria or the oxalosuccinic decarboxylase of pig heart. When parapyruvate and α-ketoglutarate were

present in equimolar concentrations, 45 per cent inhibition of isolated α-ketoglutaric oxidase was observed (Table III, Column 3); increasing the concentration of α-ketoglutarate reduced the inhibition. However, the degree of inhibition of α-ketoglutarate oxidation by intact mitochondria (Table III, Column 1), the inhibition of the ability of α-ketoglutarate to promote pyruvate oxidation (Table III, Column 2), and the inhibition of

TABLE III

Inhibition by Parapyruvate of α-Ketoglutarate Oxidation and of Ability of α-Ketoglutarate to Promote Pyruvate Oxidation

The medium is as in Table I for mitochondrial oxidation of pyruvate and α-ketoglutarate (Columns 1 and 2). For the α-ketoglutaric oxidase assay (Column 3), the following were present in final concentration: cysteine (1.67 mM), DPN (1 mM), CoA (0.1 mM), and glycylglycine buffer (pH 7.2, 33 mM). The oxidase preparation was diluted to contain 5 γ of protein per ml. and 0.01 ml. used. Change in optical density at 340 mμ recorded; temperature was 30°.

Parapyruvate	α-Ketoglutarate	Per cent inhibition		
		Oxidation of α-ketoglutarate (1)	Ability of α-ketoglutarate to promote pyruvate oxidation (2)	α-Ketoglutarate-oxidase (3)
mM	*mM*			
0.1	1.0	0.0		
0.3	1.0	2.3		
1.0	1.0	10.8	12.5	
5.0	1.0	64.0	60.0	
1.0	0.05		87.0	
1.0	5.0		3.5	
5.0	5.0		19.5	
0.35	1.67			13.0
1.67	1.67			45.0
16.7	1.67			84.5
1.67	16.7			25.1

the isolated oxidase were all much less than would be anticipated from the marked inhibition of the tricarboxylic acids to promote pyruvate oxidation (Table II), especially in view of the accumulation of α-ketoglutarate to concentrations a good deal in excess of the inhibitor present. Analysis of mitochondria and medium at the termination of an experiment showed that essentially all the α-ketoglutarate was present in the medium and hence that accumulation was not intramitochondrial.

It was obvious that, when α-ketoglutarate and parapyruvate were added simultaneously to the mitochondria, little inhibition of α-ketoglutarate

oxidation was observed, but, if the inhibitor were allowed access to the mitochondria *before* α-ketoglutarate was produced in the system (from added tricarboxylic acids or from pyruvate in the presence of malate), potent inhibition was obtained and α-ketoglutarate accumulated in quantities equivalent to the pyruvate or tricarboxylic acid utilized. When the mitochondria were preincubated with parapyruvate for varying periods, it was found that the oxidation of subsequently added α-ketoglutarate was strongly inhibited. Likewise, inhibition of the ability of α-ketoglutarate to promote pyruvate oxidation, quite comparable to that observed with

TABLE IV

Inhibition of Ability of α-Ketoglutarate to Promote Pyruvate Oxidation by Different Concentrations of Parapyruvate Preincubated with Mitochondria for Various Times

Pyruvate is at 5 mM and α-ketoglutarate at 1 mM; medium as in Table I. The mitochondria were incubated with parapyruvate and pyruvate at 27° and the α-ketoglutarate tipped in at the times indicated.

Parapyruvate	Preincubation time	Per cent inhibition	
		Q_{O_2} 10 min.	Q_{O_2} 60 min.
mM	*min.*		
0.01	20	34	5
0.03	20	57	26
0.1	20	98	86
0.3	20	99	97
1	20	100	99
1	15	92	81
1	10	68	61
1	5	57	57
1	2	47	55
1	0	21	54

the tricarboxylic acids, was observed after preincubation with parapyruvate, as shown in Table IV. When the preincubation period was 20 minutes, marked inhibition was seen with as low a concentration as 0.1 mM and definite inhibition with 0.01 mM. There was no deterioration of the mitochondria in the control vessels during these preincubation periods in the absence of a cycle intermediate.

Mechanism of Inhibition—Entry of parapyruvate into the reaction sequence of the oxidase appeared possible. Decarboxylation of parapyruvate by intact mitochondria and an acetone powder from pig heart were shown by determinations of CO_2 evolution (an initial Q_{CO_2} of 150 with mitochondria). Decarboxylation, followed by oxidation, of parapyruvate yields citramalate. Citramalate itself, however, is not the active inhibitor since

at 1 mM it inhibited the ability of citrate to promote pyruvate oxidation only 20 per cent, whereas the same concentration of parapyruvate inhibited completely. This indicated that parapyruvate was not oxidized after decarboxylation (this was substantiated by the fact that no oxygen uptake was ever observed when parapyruvate was added alone to mitochondria) but remained as a citramalyl derivative.

By comparing initial and later inhibitions in Table IV, it is seen that some recovery occurred from partial inhibition produced by lower concentrations of parapyruvate. It was also found that the block in the cycle could be overcome to a certain extent when the accumulated α-ketoglutarate had arisen to a sufficiently high concentration. Thus when all of the pyruvate initially supplied to the mitochondria had been oxidized in the

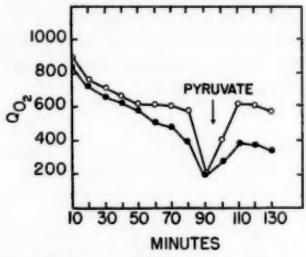


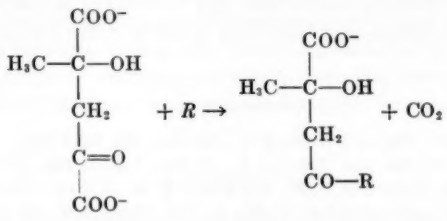
FIG. 4. Effect of pyruvate addition after utilization of pyruvate initially present; medium and conditions as in Table I. Oxidation of pyruvate (3 mM) with malate (2 mM) allowed to proceed until sudden drop in Q_{O_2} indicated depletion of pyruvate; pyruvate (3 mM) tipped in at 95 minutes. ○, control with pure pyruvate; ●, with parapyruvate (1 mM).

presence of parapyruvate and converted mainly to α-ketoglutarate, the Q_{O_2} fell to a low level at which it remained for several hours, the respiration not failing completely as would be expected in a system depleted of pyruvate. Additional pyruvate, tipped in during this low level period of respiration, immediately increased the Q_{O_2} to a value near that of the control vessel without inhibitor and to which pyruvate had been similarly added (Fig. 4). Hence some substances must have been present which could form oxalacetate for condensation with the pyruvate added later.

DISCUSSION

Parapyruvate is a potent inhibitor of the oxidative decarboxylation of α-ketoglutarate to succinate in rat heart mitochondria and has no apparent action at the concentrations used on any other reaction in the cycle. Parapyruvate is structurally similar to α-ketoglutarate, and it would not be surprising if competition could occur between these two substances for the

substrate-binding site on the α-ketoglutaric oxidase. No direct competition of this nature could be demonstrated, however. The necessity of preincubation of the mitochondria with parapyruvate for potent inhibition of the α-ketoglutaric oxidase, the demonstrated decarboxylation of parapyruvate by mitochondria, the inability of the mitochondria to oxidize parapyruvate, and the weak inhibitory action of citramalate all point to a mechanism of inhibition in which parapyruvate enters into the reaction sequence of the α-ketoglutaric oxidase but is unable to be oxidized, thus remaining as a citramalyl derivative that blocks the utilization of α-ketoglutarate. In the presence of α-ketoglutarate, parapyruvate cannot be acted upon by the oxidase as readily, and the inhibition produced develops slowly. It is a competitive inhibition in its development but, once the inhibition is established, it is reversible with difficulty. The reaction of parapyruvate by which the α-ketoglutaric oxidase is blocked may be written provisionally as



in which *R* refers to that component of the enzyme system forming the citramalyl intermediate.

Since the inhibitor has been demonstrated in relatively large amounts in pyruvic acid that was left standing for any length of time after distillation, in most commercial samples of sodium pyruvate and in sodium pyruvate prepared by the standard procedure (11), proper precautions should be taken in certain metabolic studies which utilize pyruvate. The presence of sufficient quantities of inhibitor in pyruvate can be detected by the polybromoacetone color reaction as used in the determination of citrate (12), but the most sensitive method at present would be a determination of the inhibition of the abilities of citrate or isocitrate to promote pyruvate oxidation in a heart mitochondrial preparation. Preliminary studies have shown that inhibition is produced in mitochondria from tissues other than the heart, but further work must be done to compare the degrees of inhibition accurately. It is possible that pyruvate containing the inhibitor was used in certain previous investigations on pyruvate oxidation (13–17) where α-ketoglutarate was found to accumulate or citrate was unable to initiate pyruvate oxidation. We should like to emphasize that, when pyruvate is used at 5 mM or at higher concentrations, the presence of the inhibitor at

as little as 1 per cent in the pyruvate will produce inhibition above 50 per cent in the α-ketoglutaric oxidase under the appropriate conditions.

SUMMARY

Parapyruvate inhibits specifically the oxidative decarboxylation of α-ketoglutarate to succinate in rat heart mitochondria and consequently produces a block in the cycle so that pyruvate is unable to be oxidized in the normal manner. The presence of approximately equimolar concentrations of α-ketoglutarate depresses the development of the inhibition, and thus preincubation with parapyruvate is necessary to demonstrate the potent inhibitory activity of parapyruvate. Evidence is presented that the mechanism of inhibition involves the entry of parapyruvate into the reaction sequence of the α-ketoglutaric oxidase, forming an acyl derivative that is not oxidized and hence blocks the enzyme. Parapyruvate occurs as an impurity in most commercial and laboratory-prepared sodium pyruvate, and the importance of this is discussed in relation to certain metabolic studies.

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METABOLIC STUDIES ON HEART MITOCHONDRIA*

III. THE ACTION OF PARAPYRUVATE ON α-KETOGLUTARIC OXIDASE

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Studies on rat heart mitochondria have shown that parapyruvic acid, the dimer of pyruvic acid, is a potent inhibitor of the tricarboxylic acid cycle, producing its effect by a specific block of the α-ketoglutaric oxidase (1). The present study attempts to elucidate the mechanism of this inhibition on the α-ketoglutaric oxidase isolated from heart. The evidence to be presented indicates that parapyruvate inhibits the oxidase by entering into the sequence of reactions associated with the operation of this enzyme complex and blocking this sequence by depletion of a necessary cofactor.

EXPERIMENTAL

The α-ketoglutaric oxidase was prepared from pig heart and purified as described by Sanadi *et al.* (2). The enzyme preparation contained 1.71 mg. of protein per ml. and had a specific activity of 234. The oxidase activity was determined by two methods. The "complete" assay, modified from the method of Hift *et al.* (3), involved determination of the rate of DPN[1] reduction spectrophotometrically in the presence of added CoA. The "incomplete" assay, according to the method of Sanadi and Littlefield (4), involved determination of the rate of reduction of the dye 2,6-DCPIP. It was found that neither magnesium nor DPT addition was required in either test; nevertheless, magnesium was added in the complete assay and both were added in the incomplete assay as specified in the original descriptions of the procedures. All determinations were made at 30° in a Beckman model DU spectrophotometer with constant temperature attachment.

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[1] The following abbreviations are used: DPN, diphosphopyridine nucleotide; CoA, coenzyme A; DPT, diphosphothiamine; 2,6-DCPIP, 2,6-dichlorophenolindophenol.

Parapyruvate was prepared from the lactone of the dimer of pyruvic acid by allowing an aqueous solution of the lactone at pH 7.0 to stand overnight at room temperature, during which time the lactone ring opened and equilibrium was established (approximately 76 per cent parapyruvate). This solution was reneutralized immediately before use. α-Ketoglutaric acid was recrystallized as described previously (5) and neutralized immediately before use with KOH. CoA was 75 per cent pure and DPN 90 per cent pure. Lipoic acid was kindly supplied by Dr. E. L. R. Stokstad (Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York) and *l*-citramalic acid, isolated from apple peel, was generously supplied by Dr. A. C. Hulme (Department of Scientific and Industrial Research, Food Investigation, Ditton Laboratory, Maidstone, England).

Results

Action of Lactone—Inasmuch as, at equilibrium, the solution of parapyruvate contained approximately 24 per cent of the lactone, it was important to determine whether the lactone had any effect on the enzyme; it had not had any measurable action on the cycle in heart mitochondria (1). The initial rate of opening of the lactone ring is rather high, and thus the lactone solution was prepared so that it could be added immediately to the enzyme system. It was very important to adjust the pH of the lactone solution to 7.4 since the buffering capacity of the test medium was not adequate to maintain the pH; if this is not done, inhibition of the enzyme occurs because of the low pH. The lactone (5 mM) added immediately before determination of DPN reduction by the enzyme in the presence of α-ketoglutarate (5 mM) in the "complete" assay was found to have no initial inhibitory action, but, after 4 minutes, slight inhibition was observed which reached about 7 per cent at 10 minutes, indicating the opening of the lactone ring. When the lactone (5 mM) was incubated for 20 minutes with the oxidase before the test, inhibition of 40 per cent was observed in the initial rate. This degree of inhibition would be equivalent to that produced by 0.02 mM parapyruvate under the same conditions (1), and thus the ring opening of less than 0.5 per cent of the lactone would account for the observed results. It seems likely therefore that the lactone itself was not inhibitory, even in relatively high concentration.

Effect of Preincubation of Enzyme with Parapyruvate—The degree of α-ketoglutaric oxidase inhibition by parapyruvate in mitochondria depended upon the length of time the mitochondria were exposed to the inhibitor before α-ketoglutarate was added to, or produced within, the system (1). By using the "complete" assay, a similar time relationship was found for inhibition of the oxidase (Fig. 1). In each case a control was used in which the enzyme without parapyruvate was allowed to stand at the same tempera-

ture and time interval, since the activity of the enzyme decreased slowly when brought to 30°. The preliminary incubation time required for maximal inhibition varied with the parapyruvate concentration, lower concen-

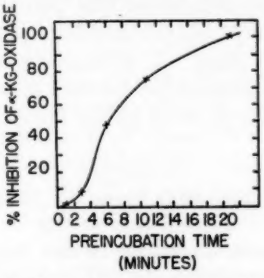

FIG. 1. Effect of preincubating the enzyme for various times with parapyruvate. "Complete" assay system as in Table I; preincubation at 30°. The final concentration of parapyruvate was 1 mM.

TABLE I

Inhibition of α-Ketoglutaric Oxidase by Parapyruvate after Preincubation of Enzyme and Inhibitor

"Complete" assay system containing, in final concentration, CoA (0.3 mM), DPN (1 mM), cysteine (10 mM), $MgCl_2$ (10 mM), potassium phosphate buffer (pH 7.4, 0.1 M), and α-ketoglutarate (5 mM). Cysteine and CoA were incubated in a cuvette for 3 minutes; other components were added and, to start the reaction, 0.1 ml. of enzyme solution was diluted 1:10 with phosphate buffer (pH 7.2, 0.02 M). The enzyme was previously incubated with various concentrations of parapyruvate for 25 minutes at 30°. The controls contained enzyme previously incubated with phosphate buffer; a change of optical density at 340 mμ was recorded.

Preincubation concentration of parapyruvate	Initial inhibition	10 min. inhibition
mM	*per cent*	*per cent*
0.025	36	9
0.25	78	41
2.5	91	58
25	100	98

trations requiring a longer period. Parapyruvate concentrations as low as 0.005 of the α-ketoglutarate concentration produced appreciable inhibition of the oxidase if allowed a sufficiently long period of previous incubation with the enzyme, as shown in Table I.

Action of Parapyruvate in Incomplete Assay—When 2,6-DCPIP was used as the electron acceptor for the oxidative decarboxylation of α-ketoglutarate, succinate was formed (not succinyl CoA as in the "complete"

assay), and presumably lipoic acid, CoA, and DPN were not involved. Inhibition of the initial decarboxylation step by parapyruvate could thus be tested in this assay. The results are given in Table II. It may be seen, in confirmation of previous deductions, that parapyruvate was attacked by the oxidase and oxidatively decarboxylated at approximately the same rate as α-ketoglutarate in this assay. It is evident that inhibition was small if it occurred at all. Some competition between α-ketoglutarate and parapyruvate would be expected to occur as the enzyme approached substrate

TABLE II

Effect of Parapyruvate in Incomplete Assay System for α-Ketoglutaric Oxidase

"Incomplete" assay system containing, in final concentration, DPT (1 mM), $MgCl_2$ (10 mM), potassium phosphate buffer (pH 7.4, 0.1 M), and sufficient 2,6-DCPIP to give an optical density of about 0.400. The reaction was started with 0.1 ml. of enzyme solution diluted 1:10 with phosphate buffer. Parapyruvate was either added to the basic medium or previously incubated with the enzyme for 10 minutes at 30°. Controls were made with enzyme previously incubated with phosphate buffer where necessary. Change in optical density (E) at 600 mμ recorded; reaction time, 1 minute.

Conditions	α-Ketoglutarate	Parapyruvate	ΔE_{600}
	mM	*mM*	
No previous incubation	10	0	57
	0	5	35
	0	10	53
	10	5	79
Previous incubation	10	0	44
	0	5	27
	0	10	41
	10	5	53

saturation. Preincubation of the enzyme with parapyruvate for 10 minutes did not alter these relations.

Localization of Inhibition—Parapyruvate itself in the "complete" assay did not bring about reduction of DPN and was not oxidized. Since the results with the "incomplete" assay showed that the enzyme could decarboxylate parapyruvate and since decarboxylation was observed by a mitochondrial preparation (1), it is likely that parapyruvate was decarboxylated in the "complete" assay to form a citramalyl-lipoic acid, analogous to the formation of succinyl-lipoic acid from α-ketoglutarate; however, the transacylation reactions by which reduced lipoic acid is released for reduction of DPN did not occur. Such an entry of parapyruvate into the enzyme sequence would also explain the necessity for preliminary incubation to

produce marked inhibition. Formation of a stable citramalyl derivative with either lipoic acid or CoA would deplete the system of a necessary component and prevent the oxidation of α-ketoglutarate. The activity of the oxidase in the "complete" assay was completely dependent on added CoA. If the stable citramalyl derivative contained CoA, addition of more CoA after the block had been produced would be expected to overcome the inhibition; however, addition of extra CoA at the beginning of the reaction or after the block had been established did not alter the development or degree of inhibition. Additions of excess DPT or lipoic acid, either before or after inhibition was established, also had no effect on the pattern of inhibition; it is probable that DPT and lipoic acid are bound tightly to the enzyme so that additional DPT or lipoic acid cannot reach the active sites on the enzyme. It is also possible that a substance other than DPT or lipoic acid is involved. The possibility that citramalate, formed from parapyruvate, might be the true inhibitor was tested by preincubation of the oxidase with citramalate (5 mM) for 10 minutes in the "complete" system, but only 6 per cent inhibition was observed, confirming the results in mitochondria (1).

Action of Arsenite on Oxidase—Arsenite is believed to react with the sulfhydryl groups of lipoic acid (6) and may be used to evaluate the importance of these groups in the α-ketoglutaric oxidase. Arsenite (3.3 mM) has been shown to inhibit an impure α-ketoglutaric oxidase 55 per cent (7). The purified oxidase was found to be much more sensitive; in the "complete" system, arsenite at concentrations as low as 0.005 mM produced detectable inhibition, and 50 per cent inhibition occurred at 0.03 mM. This inhibition was rapid and was not increased by preliminary incubation of the enzyme with the arsenite. In the "incomplete" assay, with the dye, no inhibition was found even with 0.5 mM arsenite which was sufficient to inhibit the "complete" system almost 100 per cent.

Effect of α-Ketoglutarate on Development of Inhibition—The presence of α-ketoglutarate depressed the rate at which parapyruvate inhibited the cycle in heart mitochondria (1). When α-ketoglutarate and parapyruvate were present in equimolar concentrations (5 mM) in the "complete" system, inhibition slowly increased over 10 minutes, indicating that parapyruvate could enter the enzyme sequence in the presence of substrate; however, the rate of entry was much reduced, since at this concentration complete inhibition would have been produced in 10 minutes of preliminary incubation with the enzyme in the absence of substrate, whereas with substrate the inhibition reached only 38 per cent. Similar results were obtained with 0.5 mM parapyruvate and 5 mM α-ketoglutarate; the inhibition at 10 minutes was only 19 per cent, whereas without substrate it would have been 60 per cent.

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Reversal of Inhibition—The evidence for reversibility of the inhibition in mitochondria (1) was confirmed with the oxidase (Table I). If the parapyruvate concentration was not too high, a fair degree of reversal of inhibition was observed in the presence of α-ketoglutarate, indicating that the citramalyl intermediate may be dissociable under certain conditions.

DISCUSSION

Parapyruvate is a structural analogue of α-ketoglutarate and reacts with the substrate site on the α-ketoglutaric oxidase; however, it does not remain bound to this site to block off the substrate, but is decarboxylated in the same manner as α-ketoglutarate to block the sequence of reactions by forming a relatively stable intermediate depleting the system of a necessary cofactor. The evidence that this intermediate is probably citramalyl lipoic acid is as follows. The sequence of reactions postulated by Gunsalus (8) for acyl generation from α-keto acids may be assumed for the "complete" oxidase assay system: decarboxylation of α-ketoglutarate (involving DPT), transfer of succinyl group to lipoic acid, transfer of succinyl group from lipoic acid to CoA, and oxidation of the reduced lipoic acid by DPN. Parapyruvate presumably undergoes the first two reactions, but the transfer of the citramalyl group to CoA does not occur since no evidence was found for reduction of DPN with parapyruvate in the "complete" assay system, and addition of excess CoA did not restore activity. There is a possibility that the stable intermediate may be a citramalyl derivative of some unknown acyl acceptor instead of lipoic acid. For the "incomplete" oxidase assay system, the following reactions may be assumed: decarboxylation of α-ketoglutarate (involving DPT) and reduction of the dye with formation of succinate. Parapyruvate is oxidatively decarboxylated by this system but does not inhibit it because no acyl intermediate with lipoic acid is involved.

Lipoic acid remains bound to the enzyme during the isolation of α-ketoglutaric oxidase, and it is not surprising that the citramalyl lipoic acid would also remain bound. This is substantiated by the fact that addition of excess lipoic acid did not overcome the inhibition. The difficulty would seem to be the transfer of the citramalyl group from lipoic acid to CoA. It is known that the lipoic transacylase that catalyzes this transfer shows a fair degree of specificity (9); thus the transfer of a β-hydroxy-β-methylsuccinyl group might be impossible. If the terminal carboxyl group is involved in the transfer of the acyl group, it might be supposed that the hydroxyl and methyl groups adjacent sterically interfere in the reaction. The postulated block in the system is diagrammatically shown in the lipoic acid cycle as formulated by Gunsalus (9) in Fig. 2.

The postulated mechanism accounts for the high inhibitory potency of

parapyruvate since only a sufficient amount to deplete lipoic acid from the system would be required. Arsenite probably inhibits the oxidase by forming a stable compound with the sulfhydryl groups of lipoic acid and, since the two inhibitors may block the oxidase at the same site, it is interesting that they are of approximately equal inhibitory potency, 0.02 to 0.03 μmole of each in the 1 ml. of test mixture producing about 50 per cent inhibition.

We have preliminary evidence that parapyruvate also inhibits the α-ketoglutaric oxidase in tissues other than the heart. Mitochondrial and tissue slice studies have shown inhibition in brain and kidney, while the isolated oxidase from *Azotobacter* was inhibited almost as readily as the heart enzyme.

Several problems arise with respect to the action of parapyruvate on intact cells, isolated tissues, or whole animals. In the first place, it is possible that permeability to the inhibitor may limit the degree to which it will

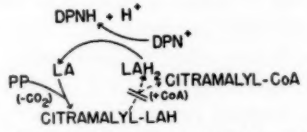

FIG. 2. Diagrammatic representation of the block produced by parapyruvate in the lipoic acid cycle. The reactions are represented as formulated by Gunsalus (9). The reactions represented by broken arrows are postulated not to occur, and hence the cycle is blocked at the point indicated. LA = lipoic acid; PP = parapyruvate.

penetrate certain cells, since both carboxyl groups have rather low pK values (2.35 and 3.95), and at physiological pH very little would exist in the undissociated forms. Secondly, the degree to which the parapyruvate will enter into the α-ketoglutaric oxidase will depend on the activity of the cycle and the concentration of α-ketoglutarate present. Thirdly, in certain tissues there may be enzymes capable of attacking parapyruvate and removing it. Fourthly, the degree of effect on the function of a particular cell or tissue will depend on the energy requirements from the cycle and the α-ketoglutarate-succinate step, and possibly upon the effects of accumulated α-ketoglutarate if the block were established.

SUMMARY

The mechanism by which parapyruvate inhibits α-ketoglutaric oxidase has been investigated, and it is postulated that a block in the reaction sequence is produced by the formation of a stable citramalyl-lipoic acid, depleting lipoic acid from the enzyme system. α-Ketoglutarate competes with parapyruvate during the development of the inhibition but only with difficulty after the inhibition has been established.

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A MICROCOLORIMETRIC METHOD FOR THE DETERMINATION OF 1,2-PROPANEDIOL PHOSPHATE*

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Recent interest in the metabolism of 1,2-propanediol phosphate (1–9) has made desirable the development of a specific assay procedure for the determination of this compound in tissues. The extreme stability of the phosphorylated compound to strong acid and to alkali (1, 10) and its ready hydrolysis to 1,2-propanediol and phosphate by both acid and alkaline phosphatase (1) have led to the development of an assay procedure based on the oxidation of 1,2-propanediol to acetaldehyde and formaldehyde by periodate (11). An aeration train has been utilized by this method similar to that described by Shinn and Nicolet (12), in which the aeration is carried out at pH 7.0 to 7.2 and thereby acetaldehyde is selectively separated from formaldehyde and trapped in a solution of sodium bisulfite. Acetaldehyde is then condensed with *p*-hydroxydiphenyl in concentrated sulfuric acid as described by Eegriwe (13) and its concentration determined colorimetrically.

EXPERIMENTAL

Reagents—Sodium periodate, 80 μmoles per ml., sodium salt being preferred to the potassium salt because of its greater solubility in water. Phosphate buffer, 1.0 M, was prepared in the usual manner and the pH adjusted to 7.1; an aqueous sodium bisulfite solution, 5 per cent, was prepared daily. An alkaline solution of *p*-hydroxydiphenyl was prepared according to previously described procedures (14), and sulfuric acid, c.p., and water-pumped nitrogen were used in the analytical method to be described. Partially purified intestinal alkaline phosphatase was prepared by the method of Schmidt and Thannhauser (15). The purification was carried up to the last step (15) and allowed to remain in 0.02 N ammonium

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acetate buffer. The phosphatase preparation was divided into 5 ml. aliquots and kept frozen until needed. At this time, 5 ml. aliquots of enzyme were diluted to 40 ml. with Veronal buffer, pH 9.6.

Methods

Acetaldehyde—Acetaldehyde was determined by the method of Eegriwe (13) as modified by Barker and Summerson (14), except that the copper-lime purification was unnecessary in this case and therefore omitted.

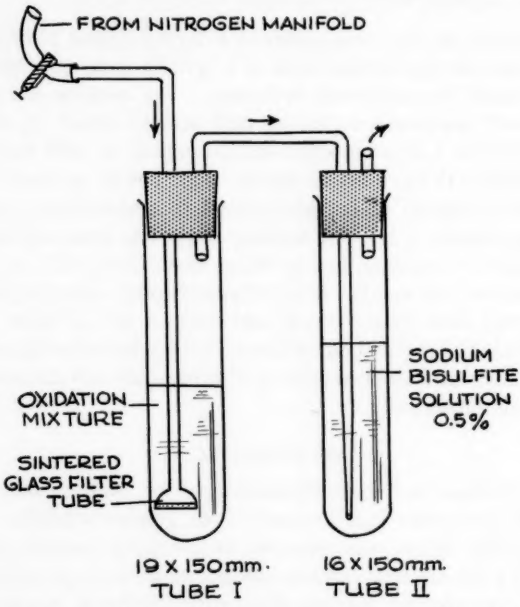

FIG. 1. Apparatus used for selective separation of acetaldehyde from formaldehyde.

1,2-Propanediol[1]—An oxidation-aeration train was constructed as depicted in Fig. 1. Eight of these units were set up in parallel manner for multiple analyses, all eight being connected to a manifold which in turn was connected to a cylinder of nitrogen. Since the filter sticks used for dispersing the gas were of varying porosity, it was necessary to fit each unit with a screw clamp for regulating the flow of nitrogen. An aliquot of 1,2-propanediol solution, pH 7.0 to 7.2, in a volume of not more than 4 ml.

[1] The following abbreviations are used throughout this paper: PD, 1,2-propanediol; PDP, 1,2-propanediol phosphate.

was added to Tube I. In order to get reproducible results, it is desirable to have at least 0.1 μmole of 1,2-propanediol. 3 ml. of phosphate buffer (1.0 M, pH 7.1) were added, followed by 1 drop of Dow-Corning antifoam A.

1 ml. of 5 per cent sodium bisulfite solution was added to Tube II, and then 9 ml. of water. Lastly, 0.5 ml. of sodium periodate solution was added to Tube I, and immediately thereafter each unit (Tubes I and II) was completely assembled and aeration with nitrogen begun. The aeration proceeds at a rapid rate under a nitrogen pressure of approximately 5 pounds per sq. in. for 2 hours. Acetaldehyde was trapped in the sodium bisulfite solution and determined by the method outlined above. The data presented in Table I were obtained by the above procedure.

TABLE I

Quantitative Determination of 1,2-Propanediol

Sample No.	PD added	PD found	Per cent recovery*
	μmoles	*μmoles*	
1	7.54	6.75	91
2	7.54	6.94	94
3	7.54	6.61	89.5
4	3.86	3.42	89
5	3.86	3.71	96.5
Average recovery			92

* Micromoles found per micromoles added × 100.

1,2-Propanediol Phosphate—5 ml. aliquots of a solution, pH 8 to 9, containing 1,2-propanediol phosphate (not more than 2 μmoles[2]), were added to a series of test-tubes. 3 ml. of Veronal buffer (0.1 M, pH 9.6) were added to the tubes containing the sample, followed by 0.5 ml. of magnesium chloride (15 per cent). 0.5 ml. of a solution of partially purified intestinal alkaline phosphatase was added and the tubes were incubated for 3 hours at 43° in a constant temperature water bath. An additional 0.5 ml. of the phosphatase solution was added and the incubation continued for another 3 hours, making the total incubation period 6 hours. Phosphatase activity was then stopped by addition of 0.5 ml. of 100 per cent trichloroacetic acid. Each sample was set up in duplicate incubation tubes and enzyme activity was stopped at zero time in 1 tube of each pair. Both tubes were then incubated for 6 hours as described above. Proteins were removed from the incubation tubes by centrifugation and the pH of the

[2] The activity of the phosphatase determines to a large extent the upper limits of 1,2-propanediol phosphate that one may use.

supernatant fluid adjusted to 7.0 to 7.2. Appropriate aliquots of these supernatant fluids were analyzed for 1,2-propanediol by the method described above. The *molar* concentration of 1,2-propanediol phosphate may then be determined by the following formula.

[PD after phosphatase action] − [PD before phosphatase action]
= [1,2-propanediol phosphate]

The data presented in Table II are typical of the data obtained after analysis of 1,2-propanediol phosphate by the above procedure.

TABLE II

Quantitative Determination of 1,2-Propanediol Phosphate

Experiment No.	Incubation time	PDP added	PDP found*	Per cent recovery†
	hrs.	*μmoles*	*μmoles*	
1	0	12.2	0	
1	6	12.2	10.8	88.5
2	0	18.15	0	
2	6	18.15	15.2	84
3	0	3.6	0	
3	6	3.6	3.3	92
4	0	2.0	0	
4	6	2.0	1.9	95

* Average of four experiments.
† Micromoles found per micromoles added × 100.

RESULTS AND DISCUSSION

Effect of Time on Liberation of Phosphate and 1,2-Propanediol from 1,2-Propanediol Phosphate—1,2-Propanediol was liberated by phosphatase action on PDP at a rate roughly parallel to that at which phosphate was liberated only after 4 to 6 hours of incubation with the enzyme. Unexpectedly, PD appeared to be liberated less rapidly than phosphate during short periods of incubation with phosphatase. It had been previously noted (1) that crude preparations of enzymes from the gut of the rat would liberate phosphate from the natural isomer of PDP at a rapid rate. It is assumed that the slower rate of enzyme action on *dl*-PDP exhibited by purified intestinal phosphatase may in part be due to the influence of the unnaturally occurring isomer of PDP on the enzyme. It is possible that the lack of agreement between phosphate and PD liberated in early phases of incubation may also be attributed to the effect of the unnaturally occurring isomer of PDP. Consequently, it was necessary to select a period of 6 hours incubation of the enzyme with PDP in order that complete agreement might be assured between the amount of phosphate and the

amount of PD liberated. Three aliquots of a standard 1,2-propanediol phosphate solution were incubated with intestinal alkaline phosphatase. Phosphatase action was halted by addition of trichloroacetic acid at 0, 10, 20, 30, 40, 50, 60, 120, 180, and 360 minutes. The protein-free filtrates were then analyzed for 1,2-propanediol and phosphate. Phosphate was determined by the method of Taussky and Shorr (16), and 1,2-propanediol by the method described above. The results are presented in Fig. 2.

Interfering Substances—Few compounds in tissues have been described which are known to yield acetaldehyde upon periodate oxidation. A series

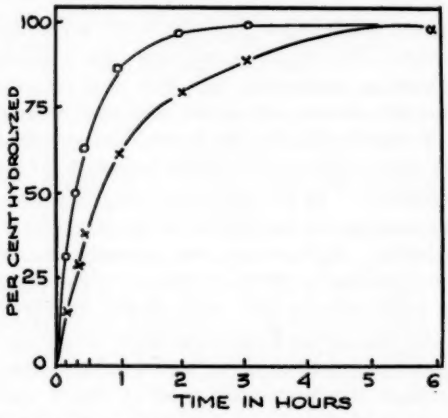

FIG. 2. The effect of time on the liberation of phosphate (○) and of 1,2-propanediol (×) from 1,2-propanediol phosphate by intestinal alkaline phosphatase.

of such compounds known to be, or suspected to be, present in tissues was submitted to analysis by the procedure for 1,2-propanediol determination. These compounds were dissolved in aqueous solution at a concentration of 10 μmoles per ml. and then 1 ml. was used in the analytical procedure. The quantity was a 50-fold excess over that used for 1,2-propanediol. The compounds investigated were 2,3-butylene glycol, malic acid, glycerol, *n*-propanol, sodium β-glycerophosphate, propionic acid, isopropanol, DL-glyceraldehyde, β-hydroxy-*n*-butyric acid, *d*(+)-trehalose, ribose, fructose-6-phosphate, fructose-1,6-diphosphate, glucose-6-phosphate, glucose-1-phosphate, rhamnose, mannose, arabinose, dulcitol, sorbitol, ethanol, pyruvic acid, acetone, dihydroxyacetone, phosphoglyceric acid, threonine, acetol, deoxyribose nucleic acid, and the ribose nucleic acids, autoclaved with 0.1 N HCl for 2 hours, DL-α-amino-*n*-butyric acid, *trans*-aconitic acid, acetylacetone, ethyl acetoacetate, methyl glyoxal, and

acetylmethylcarbinol. The phosphorylated compounds were subjected to intestinal alkaline phosphatase action for 6 hours at pH 9.6 and at 43° before they were analyzed for acetaldehyde-producing ability. Of this series of compounds, only rhamnose, 2,3-butylene glycol, acetylmethylcarbinol, and threonine gave positive results and appeared to be converted quantitatively to acetaldehyde. This interference was to be expected from their chemical structure. Rhamnose, 2,3-butylene glycol, and acetylmethylcarbinol have not been reported to be widely distributed in tissues. Threonine, an essential amino acid, has not been found in the free form in biological tissues in sufficient concentration to interfere in this determination (17–20).

Absorption curves of the color produced when acetaldehyde, formaldehyde, and a mixture of the two are condensed with *p*-hydroxydiphenyl are similar with absorption maxima at 560, 610, and 585 mμ, respectively. It was found that 60 per cent of the formaldehyde actually present in a mixture of the two aldehydes may be determined as acetaldehyde if read at 560 mμ. For this reason it was found necessary to separate the two aldehydes quantitatively. In the determination as described, care has been taken to prevent aeration of formaldehyde by controlling the pH of the solution with a buffer. If, however, the concentration of formaldehyde precursors, such as glucose or glycerol, present in the periodate oxidation tube exceeds the molar ratio of 50:1 with respect to acetaldehyde, it may be necessary to suppress the aeration of formaldehyde without suppressing that of acetaldehyde by addition of an amino acid such as glycine or alanine. This technique was previously used by Shinn and Nicolet (12). After the development of the color, it is possible to determine qualitatively whether there has been contamination by formaldehyde. Trace contamination with formaldehyde will produce a blue-violet solution, and larger amounts will completely mask the true violet color formed by acetaldehyde by producing a deep blue-colored solution. It was not found necessary to use an amino acid to suppress formaldehyde aeration except in extreme conditions, which one rarely encounters. It is not recommended for routine work; indications for its use are self-evident upon development of the color reaction.

The method described has not been used extensively in analysis for actual concentration of PD and PDP in animal tissues. However, it has been found to be quite good in determining the disappearance or appearance of these compounds in metabolic experiments with yeast cells.

SUMMARY

A microcolorimetric method has been described for the determination of 1,2-propanediol phosphate as 1,2-propanediol. The phosphorylated com-

pound is hydrolyzed to 1,2-propanediol and phosphate, after which the glycol is oxidized by periodate to acetaldehyde and formaldehyde. Acetaldehyde is selectively aerated from the mixture and collected in a bisulfite trap, and its concentration is determined colorimetrically.

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THE STRUCTURE OF RIBONUCLEASE

II. THE PREPARATION, SEPARATION, AND RELATIVE ALIGNMENT OF LARGE ENZYMATICALLY PRODUCED FRAGMENTS

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Ribonuclease consists of a single, cross-linked chain (1) containing approximately 124 amino acid residues (2, 3). The *N*-terminal sequence of this chain, together with certain deductions concerning its gross structure, has been presented earlier (1). In the present paper we wish to report on the results of one approach to the elucidation of the complete sequence of this protein.

Sanger concluded in his review in 1952 (4) that "Probably the chief need in this field is for techniques for the specific breakdown of proteins into larger peptides and for the fractionation of such peptides." Of the proteolytic enzymes available, trypsin appears to have the sharpest specificity since no evidence for peptide bond cleavage other than that expected from the fundamental studies of Bergmann and Fruton (5, 6) has been demonstrated in protein degradation studies on insulin (7) or corticotropin (8–10). Since trypsin catalyzes the hydrolysis of peptide bonds involving the carboxyl groups of the basic amino acid residues, lysine and arginine, one should obtain a mixture of fifteen peptides by complete trypsin digestion of oxidized ribonuclease, which contains 10 lysine and 4 arginine residues. This has been the basis of the approach to the structure of ribonuclease selected by Hirs, Moore, and Stein (3), who have been able to isolate and characterize thirteen peptides of distinctly different composition from ribonuclease which account for all the amino acid residues of the molecule. Eleven of these peptides contain 1 basic amino acid residue. Two of the peptides, one of which is from the *N*-terminal sequence of ribonuclease, contain 2 basic residues.

Our own approach has been to mask the lysine residues by dinitrophenylation of their ϵ-amino groups, rendering them unsusceptible to trypsin digestion, and, theoretically, allowing cleavage at the arginine residues only. There should be produced, under these circumstances, five rather large peptides from ribonuclease having an *average* size comparable to the

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peptide chains of insulin. Each of these peptides, then, should lend itself more readily to sequence studies than the much larger, intact protein. Furthermore, by the isolation from other types of digests of peptides involving arginine, and by determination of these arginine-containing sequences, the relative alignment of the large trypsin-produced peptides would be permitted. Such studies on the production, separation, characterization, and relative alignment of trypsin-produced fragments of oxidized, dinitrophenylated ribonuclease are reported below, together with ancillary data which permit the partial reconstruction of the amino acid sequence of the molecule.

Materials

Ribonuclease—As in previous studies, the present work was carried out on a single lot of commercial ribonuclease (Armour, Lot 381059), some of the chemical and physical properties of which have been described earlier (1). This preparation gave essentially a single peak on IRC-50 chromatography (11), indicating a very low initial level of the component designated as component "B."

The "B" component (or a material chromatographically similar to it) appears to be formed during storage at low temperature (Hirs, personal communication), and our working material has undoubtedly become enriched with it. The assumption has been made, however, that the chemical changes taking place during the conversion from "A" to "B" do not influence the sequence of amino acids in the molecule, but are concerned with functional groups involved in net charge and charge distribution (*e.g.* amide groups). Indeed, recent titration and spectrophotometric studies by Tanford and Hauenstein (12) indicate that the two chromatographic species differ by only a single free carboxyl group.

Performic acid oxidation of ribonuclease was performed observing the precautions outlined by Thompson (13) and by Hirs (14) to avoid the chlorination of tyrosine.

The oxidized ribonuclease was dinitrophenylated according to the basic procedure of Sanger (15).

Trypsin—The material used was commercial Armour, three times crystallized, salt-free trypsin (Lot M68009). In order to lower, preferentially, the relative activity of any chymotrypsin contaminant, the trypsin was initially subjected to an incubation in M/16 hydrochloric acid for 24 hours at 37° at a concentration of 1 per cent, as suggested by Northrop and Kunitz (16).

Results

Kinetics of Trypsin Digestion—A 1 per cent suspension of dinitrofluorobenzene-treated, oxidized ribonuclease in phosphate buffer, pH 7.9, ionic

strength 0.16, was made 0.020 per cent with respect to acid-treated trypsin and incubated at 37°. The undigested ribonuclease derivative is rendered completely soluble after about 10 minutes of digestion. Aliquots containing 10 mg. of the substrate were removed (Fig. 1) at various times, 0.1 cc. of 0.2 M dinitrofluorobenzene was added immediately, and the aliquots were dinitrophenylated according to Levy (17). After extracting the excess reagent, the samples were dried *in vacuo* and hydrolyzed for 18 hours with 6 N HCl in sealed tubes at 105°. The hydrolysates were diluted, each divided into two equal parts, extracted eight to ten times with half volumes of peroxide-free ether, and concentrated to dryness by evaporation at 60°. The ether-soluble fraction was then chromatographed according to Levy (17). The complex zones containing DNP-aspartic and DNP-glutamic acids were rechromatographed by using the *tert*-amyl alcohol-phthalate buffer (pH 5) system of Blackburn and Lowther (18). All separated DNP-amino acids were eluted and estimated spectrophotometrically at 360 mμ.

It was found in preliminary experiments that DNP-cysteic acid was not adsorbed by the acid talc-kieselguhr columns generally used for desalting solutions of water-soluble DNP-amino acids (19). The aqueous residues, after ether extraction, were therefore evaporated *in vacuo* to a dry film and DNP derivatives were dissolved away from the bulk of the salt by small washes of ethanol-0.1 N HCl (4:1). This fraction was chromatographed in two dimensions (17) and determined as above.

The absence of DNP-glycine or DNP-proline as *N*-terminal groups was demonstrated by repeating the above procedures on hydrolysates carried out in concentrated HCl, in which a portion of these otherwise highly labile derivatives survives hydrolysis (20). Correction factors for loss during hydrolysis (20)[1] and chromatography (determined independently by subjecting synthetic derivatives to the same procedures)[2] were applied, and the results were expressed as moles of DNP-amino acid per mole of bis-DNP-lysine, the *N*-terminal residue of ribonuclease.

The results of the kinetic study of digestion with acid-treated trypsin are shown in Fig. 1. Two glutamic acid terminal peptides, one aspartic acid terminal peptide, and one cysteic acid terminal peptide are formed in addition to the peptide containing the *N*-terminal bis-DNP-lysine residue already present in DNP-oxidized ribonuclease. Of the new end-groups formed, DNP-cysteic acid appears most slowly. As shown, the yield of this end-group was 0.75 mole per mole of ribonuclease at 8 hours and about 0.85 mole per mole at 24 hours. The use of trypsin which has been treated to inactivate other contaminating proteolytic enzymes is of distinct advantage. Thus it has been found in kinetic experiments similar to those sum-

[1] Recovery of DNP-cysteic acid after hydrolysis, 95 per cent.

[2] Chromatographic recovery, DNP-aspartic and DNP-glutamic acids, 90 per cent; bis-DNP-lysine, 80 per cent; DNP-cysteic acid, 90 per cent.

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marized in Fig. 1 that commercial trypsin, used at the same level of tryptic activity, led to the appearance of as much as 0.4 mole per mole of other non-specific end-groups (Ser, Thr, Ala, Val) as compared to 0.2 mole per mole (Fig. 1) after 8 hours of digestion.

In the preparation of digests for fractionation studies, a digestion time of 8 hours with acid-treated trypsin was selected. Under these conditions, non-tryptic hydrolysis was minimal and specific cleavages were essentially complete (Fig. 1).

Fractionation of Digestion Mixture

The digestion mixture was adjusted to pH 2.5 to 3.0 by the addition of hydrochloric acid, yielding an acid-soluble and an acid-insoluble fraction.

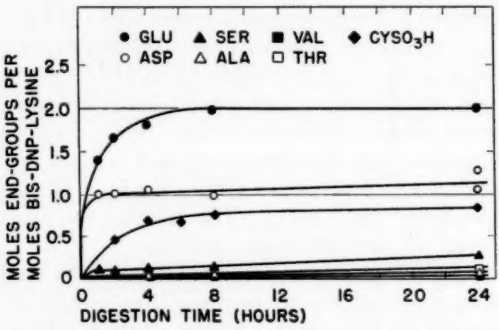

FIG. 1. Production of *N*-terminal amino acid groups during digestion of DNP-oxidized ribonuclease by acid-treated trypsin. See the text for digestion conditions.

The precipitate, containing the bulk of the material, was washed three times with 0.02 M acetic acid, and the washings were combined with the supernatant fraction.

Fractionation of Acid-Soluble Fraction

By end-group analysis the only important constituent of this fraction was found to be the aspartic acid terminal peptide. Much smaller amounts of a variety of other peptides were present, including most of the materials formed by non-tryptic hydrolysis.

The aspartic acid terminal peptide was readily fractionated from these contaminants by simple electrophoresis on paper or a cellulose powder block after the material had been desalted by adsorption on a talc-kieselguhr column (19). The migration of this component is as indicated in Fig. 2 (Band I).

Fractionation of Acid-Insoluble Fraction

Electrophoresis—The separation of the constituents of this fraction was not possible by the usual electrophoresis procedures with dilute aqueous buffers, owing to the pronounced adsorption of these compounds on the

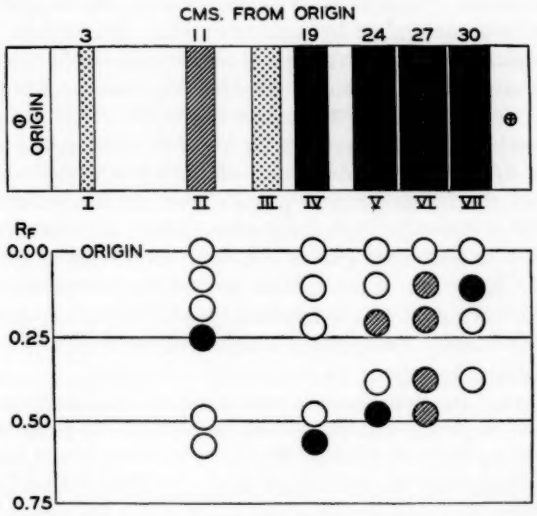

FIG. 2. Fractionation of the acid-insoluble fraction. Electrophoresis was done on Whatman No. 3 paper wet with 8 M urea-0.04 M collidine-acetate buffer, pH 7.9, and run for 12 hours at 800 volts, 12 ma., under toluene (21). Descending chromatography was done on Whatman No. 3 paper by using the system: *sec*-butanol 60, *tert*-butanol 20, 2,4,6-collidine 20, ammonium hydroxide 2, glacial acetic acid 0.25, water 100 (volume per volume). The papers were allowed to equilibrate for 2 hours in an atmosphere of the aqueous phase before introduction of the organic phase. Designation of peptides, Band I is the *N*-terminal aspartic acid Peptide C, present in trace amounts in the acid-insoluble fraction; Band II, R_F 0.27, is the *N*-terminal glutamic acid Peptide B; Band III, present in trace amounts, was not investigated; Band IV, R_F 0.54, is the *N*-terminal lysine Peptide A; Band V, R_F 0.46, is the *N*-terminal glutamic acid Peptide E; Band VI, R_F 0.22, represents unhydrolyzed Peptides C + D; Band VII, R_F 0.15, is the *N*-terminal cysteic acid Peptide D.

several types of supports tried. However, by wetting the paper (Whatman No. 3) with buffer at a pH above 7, made 8 M with respect to urea, adsorption became negligible and the components migrated in rather sharp bands, particularly when the high voltage gradient technique of Michl (21) was used. In this way, the lysine terminal peptide (Band IV) and one glutamic acid terminal peptide (Band II) were readily separated (Fig. 2).

The charge densities of the other components were sufficiently similar to prevent clean separation at this pH. At lower pH values, the components either became insoluble or were progressively more strongly adsorbed. The choice of pH range was further restricted, since high pH values had to be avoided because of the lability of the DNP derivatives in alkaline solution.

Counter-Current Distribution—Fractionation of the trypsin products could also be accomplished by counter-current distribution. The most useful solvent systems were mixtures of *sec*-butanol, *tert*-butanol, collidine, and buffers at a pH of 7 or above. The fractionation could be followed by absorption measurements at 360 mμ of the yellow DNP derivatives. A fractionation involving 150 transfers by a system composed of *sec*-butanol-*tert*-butanol-0.025 M phosphate buffer, pH 6.80 (75:25:100), allowed the separation of the lysine terminal peptide, two distinct peaks containing glutamic acid as the major end-group and one slow peak containing aspartic and cysteic acids as the end-groups. A complex mixture of peptides, containing about 20 per cent of the total sample, remained in the first few tubes. Except for a portion of the *N*-terminal lysine component, none of these fractions was found, by end-group analysis, to be of sufficient purity for analysis without preliminary electrophoretic separation.

The pattern of fractionation was very similar when distribution chromatography was employed with either cellulose columns or paper sheets. In view of the simplicity afforded by the use of a cellulose support, distribution was subsequently carried out on paper. Although streaking was no serious problem with these systems, the use of such a support suffered from the retention of variable amounts of the peptides at the point of application. This was largely avoided by using a minimal volume of solvent (1 per cent trimethylamine) to dissolve the sample so as to make repeated applications unnecessary and by inclusion of collidine in the developing solvent. A diagram summarizing the chromatographic purification of the electrophoretic components is shown in Fig. 2.

A combination of the electrophoretic and distributive chromatographic techniques permitted the clean fractionation of all five peptides present in the digestion mixture.[3] Elution of the compounds from paper was best

[3] Numerous other methods were examined for their possible value in fractionation. Owing to the large size and high level of both non-polar and polar groups present in the peptides under investigation, techniques involving resin columns, anionic cellulose columns, and most paper chromatographic procedures were unusable, on account of strong adsorption effects. A gradient salting in procedure, in which high ethanol (33 to 66 per cent) concentrations, low ionic strengths (0.002 to 0.005), and a continuously changing pH (3 to 9) were employed, yielded very encouraging enrichments, up to 80 per cent, for single constituents, as reported earlier (22). The salting in solution was allowed to flow over a column of very fine glass beads upon which was precipitated the digest mixture in a highly dispersed state, after the basic procedure of Zahn and Stahl

effected by the use of an 8 M urea solution. After adsorption of the colored derivatives in the eluates on short talc-kieselguhr columns, the columns were washed with water, and the DNP peptides were eluted rapidly with ethanol-1 per cent trimethylamine (2:1) and concentrated to dryness *in vacuo*. The dry materials were stored at 0°.

Analysis of Purified Peptides—After hydrolysis with 6 N HCl under the conditions listed in Table I, the hydrolysate (or the ether-insoluble fraction of the hydrolysate in the case of a DNP peptide) was taken to dryness *in vacuo* over KOH pellets. The residue was taken up in 3 cc. of water and dinitrophenylated and chromatographed according to Levy (17).[4, 5]

The peptides as isolated in the previous section (Fig. 2) were found to be homogeneous by end-group analysis. Peptides A and C in Table I were analyzed by using the same DNP peptide (dinitrophenylated according to Levy (17)) for both the end-group anaylsis and amino acid residue analysis. Duplicate samples of Peptides B, D, and E were analyzed for their end-groups and amino acid content separately.

The data in Table I (Peptide C) show that the destructive hydrolysis of the *N*-terminal DNP-aspartic acid is reflected in an increase of free aspartic

(23). Further investigations demonstrated, however, that this method did not permit complete purification of any single peptide and was therefore abandoned.

[4] The occurrence of certain unidentified artifact spots in the Levy two-dimensional chromatogram encountered in these studies deserves comment. One artifact running just slower than phenylalanine in the "toluene" direction and just slower than leucine in the "phosphate" direction must be distinguished from spots due to these amino acids themselves. An orange-colored artifact may occur in the glycine area, but is usually slightly faster than glycine in the "toluene" direction. Another orange-colored artifact may occur in the valine area, but is usually sufficiently slower in the "toluene" direction to allow separation. Two other artifact spots appear to be associated with decomposition products of ε-DNP-lysine or dinitrophenylation of these products. These occur in the neighborhood of bis-DNP-lysine and have a mobility in the "phosphate" direction equal to that of bis-DNP-lysine, but have different mobilities in the "toluene" direction. One of these is orange and has an R_F in "toluene" just slower than that in valine. Another is yellow and has an R_F in the "toluene" solvent between bis-DNP-histidine and DNP-leucine. Care must be taken not to confuse these spots with that due to bis-DNP-histidine, which moves slightly faster than the artifact spots in the "phosphate" direction.

[5] Two areas of the two-dimensional chromatograms frequently required rechromatography in the *tert*-amyl alcohol-phthalate buffer (pH 5) system of Blackburn and Lowther (18), with papers soaked in dilute buffer (0.05 M) to allow sufficient migration of DNP-aspartic acid and DNP-glutamic acid. One of these is the DNP-dicarboxylic acid area as noted above. The other is that area occupied by DNP-serine, DNP-methionine sulfone, and DNP-threonine, when DNP-methionine sulfone is a constituent. In these cases, a total density figure for the complex is determined, and the per cent contribution due to each of the components is determined after rechromatography. This fraction of the total density is then taken as the analytical value rather than the absolute figure as determined by the second chromatogram.

TABLE I

Amino Acid Analysis of Peptides Produced by Trypsin

Peptides A and C were analyzed after dinitrophenylation. Peptides B, D, and E were analyzed as the free peptides. F = Levy factor (17) except as described in the text.

Amino acid	Lysine terminal Peptide A*		Glutamic acid terminal Peptide B†		Aspartic acid terminal Peptide C‡		Cysteic acid terminal Peptide D§		Glutamic acid terminal Peptide E‖	
	$\frac{D_{360} \times F}{\text{Unit residue figure}}$	Residues assumed	$\frac{D_{360} \times F}{\text{Unit residue figure}}$	Residues assumed	$\frac{D_{360} \times F}{\text{Unit residue figure}}$	Residues assumed	$\frac{D_{360} \times F}{\text{Unit residue figure}}$	Residues assumed	$\frac{D_{360} \times F}{\text{Unit residue figure}}$	Residues assumed
Bis-DNP-Lys	0.9	1								
DNP-Asp					0.7	1				
Asp			3.1	3	1.4	1	5.9 ± 0.5	6	4.1 ± 0.2	4
Glu	2.1 ± 0.0	2	2.0	2			4.3 ± 0.1	4	3.1 ± 0.2	3
Ser	0.1		7.5	7–8			4.7	5	3.4 ± 0.4	3–4
Thr	1.0 ± 0.1	1	0.8	1	1.0	1	3.6	4	3.3 ± 0.0	3
$MetO_2$			3.2	3			0.9 ± 0.1	1		
Gly	0.2 ± 0.1		0.1				1.2 ± 0.2	1	2.1 ± 0.2	2
Ala	3.0 ± 0.2	3	2.1	2			3.2 ± 0.1	3	3.7 ± 0.3	4
Pro							1.3 ± 0.1	1	2.3 ± 0.4	2–3
Val							4.6	5	3.9 ± 0.1	4
Leu					1.0	1	2.4	2	1.7 ± 0.1	2
Ileu										
Phe	1.1 ± 0.0	1					1.0	1	1.0	1
His			1.0	1			0.5 ± 0.2	1¶	1.1 ± 0.2	2¶
Lys	0.7 ± 0.1	1	1.2	1	0.8	1	1.9 ± 0.5	2–3¶	1.8 ± 0.3	2–3¶
Tyr			1.0	1			1.6 ± 0.2	2¶	2.4 ± 0.6	2–3¶
$CySO_3H$**			0.9	1			4.4 ± 0.5	4–5	1.6 ± 0.2	2
Arg**	1.1 ± 0.0	1	0.9	1	1.0	1	1.0 ± 0.0	1		
Total		10		23–24		6		43–45		36–40

* Peptide A, average of two analyses performed on separate 6 N HCl hydrolysates of the DNP peptide (18 hours at 105°). The average unit residue figure, $D_{360} \times F$, based on the average value for 1 residue, was 0.217.

† Peptide B, single analysis of a 6 N HCl hydrolysate of the free peptide (18 hours at 105°). The unit residue figure, $D_{360} \times F$, based on the average value for 1 residue, was 0.218.

‡ Peptide C, single analysis of a 6 N HCl hydrolysate of the DNP peptide (18 hours at 105°). The unit residue figure, $D_{360} \times F$, based on the average leucine and threonine value, was 0.796. Absolute identification of leucine was made by chromatography of an aliquot of the hydrolysate with *n*-butanol-water (85:15) (24).

§ Peptide D, average of two analyses performed on separate 6 N HCl hydrolysates of the free peptide (22 and 70 hours at 110°), with the average deviation of the mean indicated. The average unit residue figure, $D_{360} \times F$, based on phenylalanine = 1

TABLE I—*Concluded*

residue per mole, was 0.118. Only the values obtained on the shorter hydrolysis have been taken for the notably labile residues (serine and threonine) and only those for the longer times for the amino acids which increased with longer hydrolysis (leucine-isoleucine and valine).

‖ Peptide E, average of four analyses performed on separate 6 N HCl hydrolysates of the free peptide (18 and 69 hours at 105° and 22 and 70 hours at 110°), with the average deviation of the mean indicated. The average unit residue figure, $D_{360} \times F$, based on phenylalanine = 1 residue per mole, was 0.065. Only the values obtained on the two shorter hydrolyses have been taken for the notably labile residues (serine and threonine) and only those for the longer times for the amino acids which increased with longer hydrolysis (leucine-isoleucine and valine).

¶ The values for these amino acids, present as their mono-DNP derivatives in the peptide, have given erratic low values. See the text for assignment of assumed residues.

** These values have been corrected for the 10 per cent chromatographic loss of water-soluble DNP derivatives routinely observed.

acid in the hydrolysis mixture. The sum of these aspartic acid residues gives a figure of 2.1 residues of aspartic acid in Peptide C. The value for lysine, present as ε-DNP-lysine in the peptide, gave a low value of 0.8 mole per mole when the Levy factor, 0.64, was applied. Low values were encountered in most other instances involving ε-DNP-lysine, and, therefore, a factor of 0.8, which would lead to a residue value of 1.0 for Peptide C, was taken in subsequent analyses.

The *N*-terminal lysine Peptide A is clearly a 10 residue peptide. The value for lysine is low, despite the use of the higher analysis factor as noted above, indicating an unusual lability of ε-DNP-lysine in this peptide.

The analysis for the *N*-terminal glutamic acid Peptide B indicates the presence of 23 to 24 amino acid residues. The choice between 7 or 8 residues for serine cannot be made on the basis of these data. The value for histidine has been based on the use of the factor for histidine as given by Levy (17), which measures histidine as α-DNP-histidine. The imidazole group is difficult to dinitrophenylate in *free* histidine (17), in contrast to histidine in peptidic linkage. The histidine in these peptides is in the form of imidazole-DNP-histidine. The extent of dinitrophenylation of the free α-amino group to give bis-DNP-histidine under the Levy conditions has not been investigated. Therefore, the use of this factor must be taken as a first approximation and is of value only in comparing the figures obtained for the various histidine-containing peptides.

The *N*-terminal cysteic acid Peptide D appears to contain 43 to 45 residues. Rigorous analysis of peptides of this size would require several sets of analyses, with extrapolation to zero time hydrolysis, as used by Smith and Stockell (25). The approach to structure selected here does not, however, require preliminary analyses of absolute precision, since the objective

of this approach has been to degrade the largest units (the protein as a first step) into progressively smaller specific subunits. Analyses of the smaller component peptides in which the analyses become unequivocal should later resolve the uncertainty of the analysis of the large unit.

The approximate amino acid composition of the *N*-terminal glutamic acid Peptide E indicates that this peptide does not contain arginine and must have occupied a position at the *C*-terminal end of the ribonuclease molecule. Because of its size (36 to 40 residues), the exact residue number for certain amino acids is again uncertain. The amino acids histidine, lysine, and tyrosine have given the most erratic values. These amino acids, occurring as their mono-DNP derivatives in the peptides isolated, are apparently more labile than other unsubstituted amino acid residues. Since low values for lysine were noted in the case of Peptide A, it is clear that the analyses for lysine in Peptides D and E can indicate either 2 or 3 residues. The average value of 0.5 for histidine in Peptide D, corresponding to 1 residue, suggests the assignment of 2 residues of histidine to Peptide E. These 3 histidine residues, together with the 1 residue of histidine in Peptide B, account for the 4 residues determined in the molecule by Hirs *et al.* (2).

Preliminary end-group and quantitative amino acid analysis of the constituent from Band VI, R_F 0.39 (Fig. 2), suggests that this component represents the unhydrolyzed sequence, Peptides D + E. The component from Band VI, R_F 0.22, has an aspartic acid *N*-terminal residue and probably represents the unhydrolyzed sequence, Peptides C + D.

Sequences Containing Arginine—As indicated by the kinetics of the trypsin hydrolyses discussed above, five fragments are produced during the digestion of DNP-oxidized ribonuclease in agreement with the known specificities of trypsin. One of these, the *N*-terminal fragment, may be detected by its content of bis-DNP-lysine. A second fragment, derived from the *C*-terminal end of ribonuclease, is characterized by its lack of arginine. To enable the positioning of these two peptides with respect to the remaining three requires the determination of the amino acid sequences involving 3 of the 4 arginine residues.

Acid Hydrolysis—As one approach to this problem, partial acid hydrolysates of native ribonuclease were prepared by incubation, at 37°, in 11.5 N HCl for 48 hours. After removal of HCl by repeated evaporation *in vacuo* the total digest was treated with DNFB according to Sanger (15). Excess reagent, dinitrophenol, dinitroaniline, and dinitrophenylated amino acids or small peptides not containing arginine were removed by ether extraction from the alkaline solution and after acidification. The DNP peptides insoluble in ether were then extracted into *n*-butanol and the solvent was removed *in vacuo* as rapidly as possible to avoid esterification. The

resulting mixture was streaked on Whatman No. 3 filter paper and subjected to ionophoresis at pH 3.7 in pyridine-acetic acid buffer according to Michl (21). At this pH the free carboxyl groups of aspartic and glutamic acids are only partially ionized and consequently those DNP peptides containing arginine should move towards the negative electrode. As shown in Fig. 3, four main bands were obtained along with several minor bands which were not eluted. Bands A, B, C, and D were eluted with water,

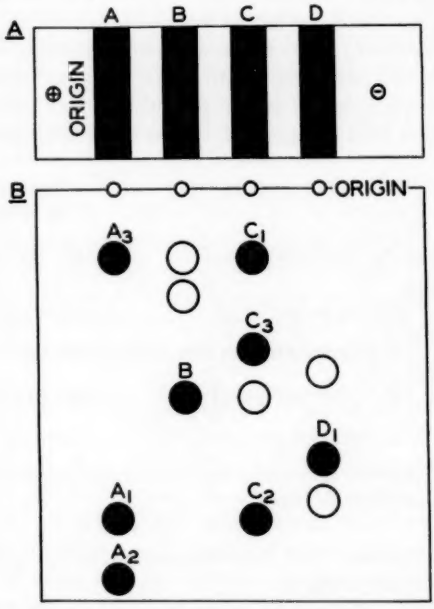

FIG. 3. Isolation of arginine-containing peptides from partial acid hydrolysates of ribonuclease. *A*, electrophoresis (21); *B*, subsequent filter paper chromatography (*sec*-butanol-3 per cent NH_3) of electrophoretic bands.

streaked on Whatman No. 3 filter paper, and chromatographed in the descending direction with *sec*-butanol-3 per cent NH_3. As shown in Fig. 3, each of the ionophoretic bands divided into several components. The major components were eluted with 6 N HCl and hydrolyzed overnight in sealed tubes at 105°. The terminal amino acid of each component (except Component B, which was not studied) was determined by two-dimensional chromatography of the ether-soluble material according to Levy (17). These chromatograms resulted in unequivocal identification and showed in each case only single end-groups. The aqueous portion of each hydrolysate

was evaporated *in vacuo* to remove HCl and the qualitative amino acid composition was determined by two-dimensional chromatography with 80 per cent phenol-1 per cent ammonia-coal gas in one dimension and butanol-acetic acid-water (4:1:5) in the second.

Fig. 4 summarizes the results of these experiments. As shown, peptides of increasing basicity were found closest to the negative electrode of the ionophoresis strips.

The acid hydrolysis studies account for two of the four arginine sequences of ribonuclease, as discussed in the section below on the reconstruction of the ribonuclease chain. The remaining two sequences were undoubtedly carried down in the large precipitate which remained insoluble in butanol following dinitrophenylation of the partial acid hydrolysates. Examination of the amino acid composition of the other chromatographic species

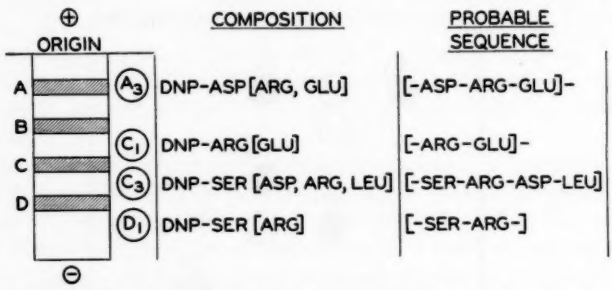

FIG. 4. The *N*-terminal amino acids and compositions of the arginine-containing peptides isolated as shown in Fig. 3.

shown in Fig. 3 indicated that they represented further hydrolysis products of the peptides listed in Fig. 4.

Pepsin Digestion—Attempts were made to isolate other arginine-containing sequences from pepsin digests of oxidized ribonuclease. A 1 per cent solution of the oxidized protein in 0.01 N HCl was incubated for 15 hours at 37° in the presence of 0.01 per cent crystalline pepsin. The solution was taken to dryness *in vacuo*. Aliquots were applied to Whatman No. 3 paper and subjected to ionophoresis (4 hours, 1500 volts) at pH 3.7 as described above (21). The pattern of ninhydrin-positive material and the location of Sakaguchi-positive components indicated the presence of four electropositive zones containing arginine. The most electropositive zone, which gave the strongest Sakaguchi test for arginine, was eluted with water, evaporated to dryness to remove pyridine acetate, and reapplied to Whatman No. 3 paper for a second ionophoresis (4 hours, 1500 volts) at pH 6.8 with the same buffer components. Five major subfractions (all migrating towards the negative electrode) were well separated and con-

tained arginine as indicated by a positive Sakaguchi test. These were eluted and analyzed qualitatively, after hydrolysis, by two-dimensional paper chromatography. These preliminary chromatograms indicated a rather complex composition for all but the least electropositive component. Small aliquots of the latter peptide were rerun at pH 3.7 and at 6.8 and appeared homogeneous under both conditions.

Acid hydrolysis of an aliquot of this peptide, followed by two-dimensional chromatography in the phenol-butanol-acetic acid system as used above, indicated the presence of the following: (Asp, Arg, Glu_2, Ala, Thr, $MetO_2$, His, Ser_{2-3}). The presence of 2 to 3 equivalents of serine and of 2 equivalents of glutamic acid per mole was suggested by the comparison with control chromatograms with differing mole ratios of the various amino acids found in the preliminary analysis of the fragment. The peptide was treated with DNFB according to Sanger (15) and, after extraction with ether at both alkaline and acid pH to remove excess reagent and soluble contaminants, the yellow reaction mixture was taken to dryness. The DNP peptide was removed from the major portion of the salt by extraction of the dry residue with glacial acetic acid, followed by rapid evaporation. Hydrolysis with HCl, followed by chromatography (17) as described above, yielded DNP-glutamic acid as the only end-group. The aqueous solution after removal of the end-group with ether contained all the amino acids found above.

Samples of the DNFB-treated peptide (1 μmole on the basis of quantitative end-group analysis (17)) prepared as described above were dissolved in 0.5 ml. of phosphate buffer, pH 8.0, ionic strength 0.15, and digested with trypsin, which was present at a level of 0.4 per cent. After incubation at 37° overnight, the solution was acidified to about pH 2 and extracted with butanol. The butanol extract was washed twice with small amounts of water and the butanol phase was taken to dryness and hydrolyzed overnight in concentrated HCl at 105°. The end-group was determined by chromatography as above and proved to be DNP-glutamic acid. The aqueous phase contained only arginine. These findings indicate that the partial structure of the peptide must be .Glu.Arg.Glu.(Asp,Ala,Thr,$MetO_2$,His,Ser_{2-3}), since arginine must be followed either by a cysteic acid or by a dicarboxylic amino acid according to the trypsin digestion data described above and since the sequence, .Arg.Asp, has already been shown to occur in another area of the protein (Fig. 4 and Table II).

The experiments above establish the presence in ribonuclease of the following arginine-containing sequences: .Ser(Arg,Asp,Leu), Asp.(Glu,Arg), and Glu.Arg.Glu.(Asp,Met,His,Ser_{2-3},Ala,Thr).

Sequence Studies; N-Terminal Aspartic Acid Peptide C—The amino acid sequence of the *N*-terminal aspartic acid hexapeptide was determined as

follows: The peptide was eluted from the electrophoretic strips and dinitrophenylated (17). The material was concentrated to dryness *in vacuo* and subjected to partial acid hydrolysis in 11 N HCl at 37° for 16 hours. After dilution with water, the solution was extracted five times with half volumes of ether, and the combined extracts were evaporated to dryness. The solution was then extracted three times with ethyl acetate, and the extracts were back-washed with water. The combined ethyl acetate extracts were evaporated to dryness. Both the ether and ethyl acetate extracts were subjected to paper electrophoresis in 0.03 M phosphate buffer, pH 7.0, at 230 volts, 10 ma., for 7 hours. The yellow, electrophoretically separated zones were eluted, desalted on a talc-kieselguhr column as described above, and hydrolyzed for 18 hours at 105° in sealed tubes. The hydrolysates were extracted with ether and chromatographed. The aqueous fractions were concentrated to dryness and chromatographed for free amino acids (26). The results giving the sequence at the *N*-terminal end of Peptide C are summarized in Table II.

TABLE II

Sequence Data for N-Terminal Aspartic Acid Peptide C

Electrophoretic zone	Distance migrated	Relative color	DNP-amino acid residue	Free amino acid residues	Composition
			N-Terminal end of Peptide C		
	cm.				
1	+23.5	1+	DNP-Asp	0	DNP-Asp
2	+18.0	2+	"	Leu	DNP-Asp.Leu
3	+15.0		Dinitrophenol	0	
4	+11.0	1+	DNP-Asp	0	DNP-Asp-NH_2*
5	+7.5	2+	"	Leu	DNP-Asp-NH_2.Leu*
			C-Terminal end of Peptide C		
1	−4.5	2+	DNP-Arg	0	DNP-Arg
2	−1.0	1+	DNP-Thr	{ε-DNP-Lys, Asp, Arg}	DNP-Thr-(Lys,Asp.-Arg)
3	+1.5	1+	DNP-Asp	Arg	DNP-Asp.Arg

* Zones 1 and 4 and Zones 2 and 5 had the same composition after complete hydrolyses. The pairs of zones which migrated more slowly towards the anode must, therefore, have been in their amide forms.

The aqueous portion of the partial acid hydrolysate was concentrated to dryness and dinitrophenylated. The solution was then extracted with

ether, acidified, and reextracted with ether and ethyl acetate as above. The material remaining in the aqueous solution, representing α-DNP-arginine and peptides involving arginine, was concentrated to dryness and subjected to paper electrophoresis as above. The yellow electrophoretic zones were eluted, desalted, and hydrolyzed with 6 N HCl at 105° for 18 hours. Each of these hydrolysates was then extracted with ether and analyzed for its *N*-terminal DNP-amino acids (17). The aqueous solution was analyzed for free amino acids (27). The results, giving the sequence at the *C*-terminal end of Peptide C, are summarized in Table II.

The sequence of Peptide C can thus be written as $AspNH_2$.Leu.Thr.Lys.-Asp.Arg.

N-Terminal Lysine Peptide A—A 1 per cent solution of Peptide A (approximately 0.5 μmole) in phosphate buffer, pH 7.9, ionic strength 0.16, was digested for 17 hours at 25° in the presence of 0.01 per cent chymotrypsin (1 per cent relative to substrate). The total digest was dinitrophenylated (17). After extraction of the excess reagent and evaporation to dryness, the residue was dissolved and subjected to paper electrophoresis in 0.033 M phosphate buffer, pH 7.01, as above. The two darkest zones were eluted, desalted, and chromatographed on paper with methyl ethyl ketone-propionic acid-water (75:25:30) (27). The eluted zones were hydrolyzed with 6 N HCl for 18 hours at 105° in sealed tubes. After ether extraction of the diluted hydrolysates, the DNP-amino acid end-groups were identified by paper chromatography (17, 18). The aqueous fractions were evaporated to dryness *in vacuo*, and aliquots were taken for free amino acid chromatography (26) and analysis according to Levy (17). The following peptides were characterized: a peptide migrating +6.3 cm. upon electrophoresis, and chromatographing with an R_F 0.77, was shown to be DNP-Glu.Arg; a peptide migrating +17.5 cm. upon electrophoresis, and chromatographing with an R_F 0.95, had the amino acid composition bis-DNP-Lys.(Glu,Thr,Ala_3,ε-DNP-Lys).Phe. In the latter peptide, phenylalanine is placed outside the parenthesis at the *C*-terminal end of this peptide in accordance with the known specificity of chymotrypsin. The *N*-terminal sequence has been previously determined (1) as Lys.Glu.Thr.-Ala. The sequence of Peptide A, occupying the *N*-terminal position in ribonuclease, can thus be written as Lys.Glu.Thr.Ala.(Ala_2,Lys).Phe.Glu.Arg. A peptide with this same composition can be constructed from data obtained on trypsin hydrolysates of oxidized ribonuclease by Hirs, Stein, and Moore (28).

Other Peptide Fragments

During the course of chymotrypsin digestion studies on the large trypsin-produced peptides described above, and from preliminary experiments on the nature of the disulfide bridges of ribonuclease, a considerable number

of peptide fragments have been isolated and analyzed. Two of these are of immediate interest in the partial reconstruction of the ribonuclease molecule as presented in a later section of this paper. A peptide liberated by chymotrypsin was isolated by filter paper electrophoresis and paper chromatography of digests of the peptide designated Peptide D. Its quantitative composition -Ser.(Leu or Ileu,Thr,$CySO_3H$,Asp).Arg. was established by two-dimensional chromatography by the method of Levy for hydrolysates of the electrophoretic zone in question. The serine end-group was determined by the usual procedure (17).

The cysteic acid-containing peptide ($CySO_3H$,Asp,Arg,Glu,Gly,Ser) was isolated as part of a disulfide "bridge" from subtilisin digests of ribonuclease, by electrophoresis according to Michl, and analyzed by two-dimensional chromatography (26) after rupture of the disulfide linkage with performic acid.

As indicated in the reconstruction given in the section below, the amino acid composition of these fragments permits the subdivision of some of the large trypsin-produced fragments into smaller groups of residues.

C-Terminal Sequence—The data permitting the reconstruction of the *C*-terminal sequence of ribonuclease are summarized below. Digestion with carboxypeptidase had earlier established the presence in ribonuclease of valine as the *C*-terminal residue (1, 29). Other residues were released during prolonged digestion. In later studies (unpublished data of Anfinsen and Richards), it was found that longer digestions with another more thoroughly diisopropyl fluorophosphate-treated carboxypeptidase preparation yielded the amino acids serine and alanine in addition to valine, suggesting the presence at the end of the chain of the grouping -(Ala,Ser).Val. Hydrazinolysis according to Ohno (30) has confirmed the presence of valine as the *C*-terminal residue. In unpublished experiments by Niu and Fraenkel-Conrat (31), partial hydrazinolysis was found to release, in addition to valine, the peptide -Ser.Val. Finally, limited pepsin digestion of native ribonuclease results in the quantitative liberation of the tetrapeptide -Asp.Ala.Ser.Val. The sequence of this peptide was established by subjecting the DNP peptide to partial acid hydrolysis. DNP-aspartic acid and DNP-aspartylalanine were isolated from the ether extract of the hydrolysate. The dipeptide DNP-serylvaline was isolated from the aqueous portion following a second treatment with DNFB. The presence of this tetrapeptide as the *C*-terminal grouping in ribonuclease is thus strongly inferred from the combined data above.

Partial Reconstruction of Ribonuclease Chain

Point 1—Ribonuclease contains, as its *N*-terminal sequence, the grouping Lys.Glu.Thr.Ala- (1) and, as its *C*-terminal sequence, -Asp.Ala.Ser.Val.

Point 2—Trypsin digestion of dinitrophenylated, oxidized ribonuclease leads to the production of five fragments labeled Peptides A, B, C, D, and E. These peptides contain the *N*-terminal and *C*-terminal amino acid residues and the amino acid groupings indicated in Fig. 5. The *C*-terminal sequence indicated for Peptide D is based on the isolation, described above, of the *C*-terminal arginine sequence from chymotrypsin digests of Peptide D.

Point 3—In view of the end-group studies, the *N*-terminal lysine Peptide A is assignable to the *N*-terminal position of ribonuclease.

Point 4—Peptide E, since it lacks arginine, must be assigned to the *C*-terminal end of ribonuclease.

Point 5—The proper relative alignment of the five trypsin fragments below is dependent on knowledge of the structure of the arginine-containing sequences which form the "bridges" between them. Three relatively small arginine-containing "bridges" have been isolated from acid and pepsin hydrolysates of ribonuclease. These "bridges" are (1) -Ser(Arg,Asp,

Peptide	Schematic Structure
A	Lys.Glu.Thr.Ala.....Glu.Arg
B	Glu.....Arg
C	Asp.Leu.Thr.Lys.Asp.Arg
D	$CySO_3H$.....Ser.(Leu or Ileu),Thr,$CySO_3H$,Asp).Arg
E	Glu.....Asp.Ala.Ser.Val

FIG. 5. Partial structures of peptides produced during trypsin digestion of DNP-oxidized ribonuclease.

Leu), (2) Asp.(Glu,Arg)-, and (3) -Glu.Arg.Glu.(Asp,Met,Ala,His,Thr, Ser_{2-3}).

Point 6—"Bridge" (3) contains methionine. The carboxyl group of the arginine residue in "bridge" (3) must join either Peptide B or E, since only these two are *N*-terminal glutamic acid peptides. Since, according to the analyses given earlier, Peptide E is devoid of methionine, arginine "bridge" (3) must constitute a juncture between Peptide B, on the right, with another of the major trypsin fragments on the left of arginine. Further, this latter peptide must have as its *C*-terminal sequence -Glu.Arg.

Point 7—As shown in Fig. 5, neither Peptide C nor Peptide D obeys this restriction. It may therefore be concluded that Peptide B is preceded by Peptide A and we may write the tentative arrangement A-B-(D,C)-E.

Point 8—Arginine "bridge" (1) could have the following alternative structures: (1) .Ser.Arg.Asp.Leu., (2) .Ser.Asp.Leu.Arg., (3) .Ser.Leu.Asp.Arg., or (4) .Ser.Asp.Arg.Leu. Structure (4) may be eliminated, since arginine must be followed by cysteic acid or by a dicarboxylic amino acid (Fig. 1). The arrangements indicated in structures (2) and (3), in which arginine is *C*-terminal, could occur only at the *C*-terminal end of Peptide

B (Fig. 5). Since (Table I) Peptide B does not contain leucine, structures (2) and (3) are impossible. The sequence of arginine "bridge" (1) must thus be Ser.Arg.Asp.Leu, as indicated in structure (1).

The isolation of the arginine "bridge," -Ser.Arg.Asp.Leu., together with the complete sequence of Peptide C, Asp.Leu.Thr.Lys.Asp.Arg, establishes the structure -Ser.Arg.Asp.Leu.Thr.Lys.Asp.Arg-, since the sequence, -Arg.Asp-, can occur only once in ribonuclease (only one *N*-terminal aspartic acid end-group is formed during trypsin digestion).

Point 9—Therefore, Peptide C cannot follow Peptide D, since the *C*-terminal sequence of Peptide D (see Fig. 5) is incompatible with the arrangement of amino acids in Point 8. (There is no serine in juxtaposition to arginine in Peptide D.)

Point 10—Since Peptide C cannot follow Peptide D, it must precede it. The correct arrangement is, therefore, A-B-C-D-E.

Lys . Glu . Thr . Ala . (*Ala*$_2$, *Lys*)*Phe . Glu . Arg . Glu* . (Asp , Thr , Ser$_3$, Met , His , Ala) . (Ala , Cys- , Tyr , Ser$_{3-4}$, Lys , Met$_2$, Glu , Asp$_3$) . *Ser . Arg . Asp . Leu , Thr . Lys . Asp . Arg . Cys-* . (Asp , Val , Pro , Phe , (Thr , *Lys)) (Asp$_3$, Glu$_4$, Ser$_4$, Thr$_2$, Met , Gly , Ala$_3$, Val$_4$, His , Lys$_{1-2}$, Tyr$_2$, Cys-$_{2-3}$. Ser) . ((Leu or Ileu) , Thr) . Cys- . Asp . Arg . Glu . (Gly , Ser) . (Asp$_3$, Glu$_2$, Ser$_{1-2}$, Thr$_3$, Ala$_2$, Pro$_{2-3}$, Val$_3$, Gly , (Leu + Ileu)$_2$, Phe , His$_2$, Lys$_{2-3}$, Tyr$_{2-3}$, Cys-$_2$) . *Asp . Ala . Ser . Val*

FIG. 6. Partial reconstruction of the peptide chain of ribonuclease. *, as discussed in the text (Point 11 under "Partial reconstruction of ribonuclease chain") Thr and Lys may also be included within this group of amino acids. This latter arrangement is compatible with the data of Hirs, Stein, and Moore (32).

Point 11—The large peptide (Lys,Asp$_2$,Arg,CySO$_3$H,Val,Pro,Thr,Phe) described earlier, containing phenylalanine and arginine but no glutamic acid, may be included in the considerations as follows:

There are 3 phenylalanine residues in ribonuclease, 1 in each of Peptides A, D, and E (see Table I). The phenylalanine residues in Peptides A and E can only exist in a sequence together with arginine, if the sequence also includes glutamic acid (Fig. 5). The peptide (Lys,Asp$_2$,Arg,CySO$_3$H,Val,-Pro,Thr,Phe) must therefore be a part of Peptide D and must include the arginine "bridge" between Peptides C and D. This bridge may then be extended as follows: .(Thr,Lys,((-Asp.Arg.CySO$_3$H-).(Val,Pro,Phe,Asp))), where the position of the threonine and lysine residues cannot be fixed with respect to the two groups of residues in parentheses since these 2 residues could derive from either Peptide C or D.

Point 12—Glycine occurs only in Peptides D and E. In view of the nature of the arginine "bridge" between Peptides C and D (Point 11), which rules out the occurrence of glycine within 5 residues of arginine, the glycine-containing peptide (CySO$_3$H,Asp,Arg,Glu,Gly,Ser) must occur at the bridge between Peptides D and E. This grouping may be further sub-

divided, in view of the *C*-terminal sequence of Peptide D (Fig. 5) as follows: .($CySO_3H$,((.Asp.Arg.Glu).(Gly,Ser))).

Point 13—On the basis of the points listed above, and the analyses listed in Table I, we may partially reconstruct the ribonuclease chain as shown in Fig. 6.

SUMMARY

The partially reconstructed formula for ribonuclease presented in this paper summarizes a large part of the results of our structure studies to date. The object of the present communication has also been to report on the feasibility and relative simplicity of the approach to structure determination which we have chosen.

The relatively incomplete structure given here already makes possible certain general observations concerning basic features of the enzyme. Most interesting, perhaps, is the amino acid composition of the *C*-terminal portion of the molecule which apparently contains 3 of the 4 (32) proline residues and 2 of the 4 histidine residues present in the protein. In view of the inactivation of ribonuclease brought about by photooxidation of histidine (33) and because of other studies (34, 35) suggesting that the active center of ribonuclease is located near the *C*-terminal end of the chain, these observations take on special interest in connection with further investigations now in progress on the structural basis of the catalytic action of ribonuclease.

The approach to the elucidation of protein structure employed in these studies involves certain features which might become of particular advantage when larger proteins (having molecular weights of 30,000 or greater) are investigated. The specificity of trypsin, combined with the relative ease of isolation of arginine "bridges" for aligning trypsin fragments, makes it likely that any protein containing reasonable quantities of arginine may be susceptible to this general method. The present studies indicate that the separation of relatively large fragments, though rich in both dinitrophenyl groups and in cysteic acid residues, offers no great difficulty when the techniques of electrophoresis in urea solutions and of chromatography in proper solvents are applied.

In concluding this preliminary report we would like to join Hirs, Stein, and Moore (32) in expressing our gratification over the valuable interchange of information which has taken place during the structural work by their group and ourselves. Although the present reconstruction of the ribonuclease molecule has purposely been made without reference to the Rockefeller Institute contributions, it is important to observe that overlapping and consistent conclusions may be drawn from both sets of data. Thanks

are due Dr. Fred Sanger for his generous hospitality and assistance to one of us (C. B. A.) during the summer of 1954 at the Department of Biochemistry, University of Cambridge, Cambridge, England.

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THE LIMITED DIGESTION OF RIBONUCLEASE WITH PEPSIN

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Until very recently, biochemists have contemplated the problem of the mechanism of enzyme action with understandable awe in view of the enormous complexity of protein structure and because of a well entrenched assumption that enzymatic activity is dependent upon an integrated system of electron transfers and rearrangements (1–4) governed by the intact covalent and secondary structure of the molecule. Recently, however, the view has begun to emerge that the phenomenon of enzyme action may, in fact, be dependent upon structural areas, within protein molecules, of relative simplicity. For example, it has been shown that portions of various biologically active protein chains can be removed or modified by proteolytic digestion without impairment of function (5–12).

It has also been possible to examine, in a fairly direct way, the effect on enzyme activity of denaturing agents which cause changes in organized secondary structure. Studies by Steinhardt (13) on pepsin, and by Ottesen (personal communication) on subtilisin have shown that these enzymes are fully active when assayed in solutions containing urea at high levels. It has also been found that ribonuclease retains its full enzymatic activity under conditions in which the ordinarily highly compact form of the protein is extensively disoriented by 8 M urea (14). Rupture of the disulfide bridges by performic oxidation yields a derivative without enzymatic activity (15), although the degree of structural disorientation of the oxidized molecule is not much greater than that of the active urea-unfolded molecule described above (14). The necessity for restricting cross-linkages in catalysis is thus strongly inferred.

In earlier studies on the action of pepsin on ribonuclease (16), evidence was obtained suggesting that an initial product was produced differing only very slightly from the native molecule both in size and shape. The progress of this reaction has now been examined in greater detail and it has been possible to isolate and characterize partially an *inactive* derivative, differing only slightly from native ribonuclease. The nature of that deriv-

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ative and of the fragments released from ribonuclease during the pepsin digestion, considered together with the earlier data on urea, permits the conclusion that the catalytic activity of the enzyme is attributable to a relatively small portion of the ribonuclease chain and that this portion, located near the *C*-terminal end of the molecule, may be protected and stabilized in urea solutions either by the presence of disulfide bridging or by particularly strong, urea-insensitive, hydrogen bonds.

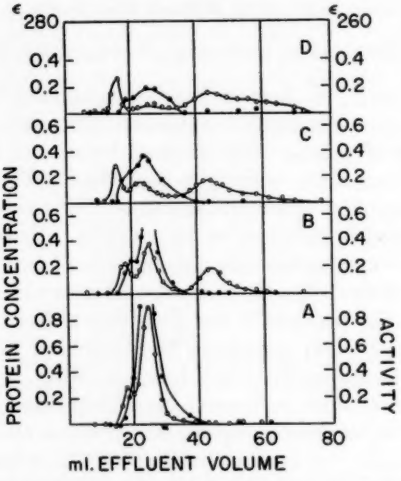

FIG. 1. Chromatography of 10 mg. samples of native and pepsin-digested ribonuclease on IRC-50 columns. Eluting buffer, phosphate, 0.2 M, pH 6.45. Digestion conditions, ribonuclease, 0.92 per cent; pepsin, 0.002 per cent; 37°, pH 1.8. Remaining ribonuclease activity, *A*, 100 per cent; *B*, 60 per cent (5 minutes); *C*, 40 per cent (10 minutes); *D*, 15 per cent (16 minutes). ●, enzyme activity (15); ○, protein concentration. The peak at 18 to 19 effluent ml. is a ninhydrin-negative artifact, arising from the resin itself.

EXPERIMENTAL

In preliminary experiments, a 0.92 per cent solution of ribonuclease (Armour, three times recrystallized) in dilute HCl (pH 1.8) was digested for varying periods of time at 37° with pepsin (crystalline). The pepsin was used at a concentration of 0.002 per cent. Activity determinations (15) were made on samples of the digestion mixture taken during the progress of the reaction (digestion being stopped by addition of phosphate buffer, pH 6.8).

A series of identical columns (17) (pH 6.45, 30 × 0.9 cm., XE-64 resin) was employed to compare the chromatographic behavior of aliquots of the

digest taken at 5, 10, and 16 minutes after the addition of pepsin with that of an initial undigested sample. Chromatography was carried out at 5°. The results of these experiments are summarized in Fig. 1. The curves show the progressive appearance of an enzymatically inactive component in the samples taken at 5 and 10 minutes. The increasing complexities of the chromatographic patterns for the 10 and 16 minute samples indicate the occurrence of further proteolytic modification at the expense of both the active enzyme and the primary inactive derivative.

Isolation and Physical Characterization of Major Inactive Component—In a large scale experiment, 200 mg. of ribonuclease were digested for 10 minutes under the conditions described above. The digest was chromatographed at a slow rate (5 ml. per tube, two tubes per hour) on a larger column of XE-64 (6 × 40 cm.) at pH 6.45, 5°, to permit good separation of

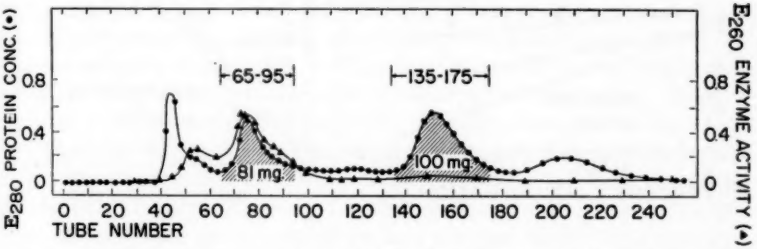

FIG. 2. Large scale chromatography of pepsin digest of ribonuclease (see the text for details).

the major inactive component from the secondary products of greater affinity for the resin. The results of this chromatogram are shown in Fig. 2. The contents of the tubes indicated in Fig. 2 were pooled and dialyzed against water to remove phosphate buffer and lyophilized. In this manner, 81 mg. of native ribonuclease and 100 mg. of the inactive material were obtained for subsequent study.

Sedimentation and Viscosity Characteristics—From previous investigations outlines in a rough way of the general size and shape characteristics of native and of performic acid-oxidized ribonuclease have been obtained (15). More recently, Harrington and Schellman have greatly extended these observations and, among other studies, have carefully examined the concentration dependence of the sedimentation and viscosity constants of both the native and oxidized molecules (18). By using these careful observations as background (see the "theoretical" lines in Fig. 3), comparisons have been made of the inactive component isolated above, both in the "native" and in the oxidized forms, with native and oxidized ribonuclease.

The points on the curves in Fig. 3 indicate, first, that the unoxidized inactive material is ultracentrifugally indistinguishable from native ribonuclease and, second, that, after oxidation, the inactive material shows less concentration dependence than the native enzyme, although the extrapolated value at infinite dilution is the same for both.

The intrinsic viscosity of the inactive substance was, within experimental error, identical with that of the native enzyme (3.3 to 3.9) and, like the latter, showed no variation with concentration. The reduced viscosity (1 per cent) of the oxidized inactive derivative (10.3) was slightly less than that of the oxidized native enzyme (12.3).

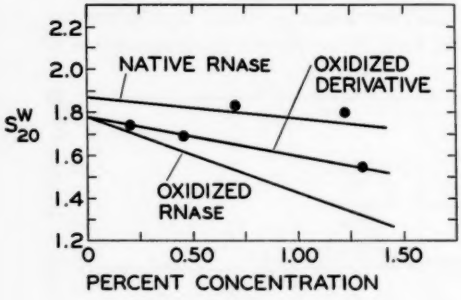

FIG. 3

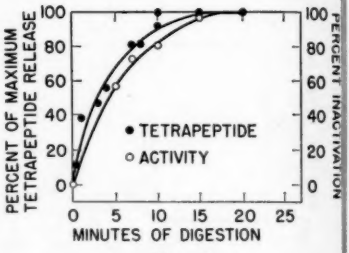

FIG. 4

FIG. 3. Sedimentation studies. Native ribonuclease, upper curve (18); inactive derivative isolated from pepsin digestion, points along upper curve; oxidized ribonuclease, lower curve (18); oxidized derivative, middle curve. All runs made in Spinco model E ultracentrifuge.

FIG. 4. Production of the peptide, Asp.Ala.Ser.Val, and loss in enzyme activity during digestion of ribonuclease with pepsin (incubation conditions as in Fig. 1).

Although no final conclusions can be drawn concerning the comparative molecular weights of the native and inactive material without further information about such factors as the extent and distribution of hydration, the above studies indicate a high degree of similarity and furnish the first suggestion as to the rather slight nature of the proteolysis involved in the initial inactivating cleavage by pepsin. They further show that *cleavage must have occurred at a point not far removed from one or the other end of the single chain of ribonuclease.*

Paper Chromatography—By paper chromatography (in butanol-acetic acid-water, 4:1:5) and by filter paper electrophoresis at pH 6.0 (19) of samples of a digestion mixture taken after different time periods, it was found that a peptide was released in progressively larger amounts. Upon hydrolysis, after elution from the chromatograms or electrophoresis strips,

the peptide was shown to contain aspartic acid, alanine, serine, and valine. This peptide, isolated earlier from more complete digests of ribonuclease, has been shown to have the sequence Asp.Ala.Ser.Val[1] (20). Since previous studies ((20), and personal communication from Fraenkel-Conrat) have shown that the *C*-terminal sequence of ribonuclease is almost certainly -Ala.Ser.Val, it may be concluded that an early event during the pepsin digestion of ribonuclease is the removal from the parent molecule of the above tetrapeptide from the *C*-terminal end of the chain.

Experiments were performed to relate the rate of appearance of the tetrapeptide fragment to the rate at which enzyme activity disappeared during pepsin treatment. The results of such studies are shown in Fig. 4. The rate of peptide production parallels, closely, the loss in activity. In these experiments, aliquots of digestion mixture were chromatographed in the butanol-acetic acid solvent system above on Whatman No. 1 filter paper in the descending direction. After location of the position of the peptide by ninhydrin treatment of guide spots, the experimental areas were cut out and the amount of ninhydrin-reacting material on the entire segment of paper was determined by the method of Cocking and Yemm (21). Control areas of paper were run concomitantly. The "leucine color equivalence" of the peptide was determined on samples before and after acid hydrolysis and in turn compared with leucine standards which had been chromatographed on the same papers for recovery control (leucine recovery ranged from 95 to 100 per cent). By using the correction factors thus obtained, it can be calculated that the appearance of the peptide is quantitatively as well as qualitatively correlated with activity loss.

These results suggest either that the loss of the tetrapeptide is *alone* sufficient to produce inactivation, or that the rate of cleavage of the bond by which this peptide is attached to the parent molecule is equaled by the rate of cleavage or modification of one or more other bonds in the ribonuclease structure which *are* responsible for loss in activity.

Determination of N-Terminal Amino Acids—Samples (both before and after performic acid oxidation) of the inactive ribonuclease derivative (Fig. 2), and of control samples of native ribonuclease, were subjected to treatment with DNFB according to Sanger (22) and also by the general procedure of Levy (23). With both methods only bis-DNP-lysine was found in subsequent two-dimensional chromatograms (23). The recovery of this end-group, after correction for destruction during acid hydrolysis,

[1] The following abbreviations are used in this and the accompanying paper: Asp, aspartic acid; Ala, alanine; Val, valine; DNFB, dinitrofluorobenzene; DNP, dinitrophenyl; $CySO_3H$, cysteic acid; Lys, lysine; Glu, glutamic acid; Thr, threonine; Phe, phenylalanine; Arg, arginine; Ser, serine; Met, methionine; His, histidine; Tyr, tyrosine; Leu, leucine; Pro, proline; Gly, glycine; Ileu, isoleucine.

was from 70 to 90 per cent of theoretical. Smaller amounts (less than 10 per cent of theoretical) of other end-groups (Ser, $CySO_3H$) were always present in the inactive component but are probably attributable to macromolecular contaminants in the chromatographic component under study (see Fig. 2) which were produced during pepsin digestion. The end-group experiments suggest that the process of inactivation by limited peptic digestion is not due to cleavage of the ribonuclease chain at any point near the *N*-terminal end. Similar results have been obtained by using the phenylthiohydantoin technique of Edman (24).

DISCUSSION

The "active center" of ribonuclease appears to involve only a relatively small portion of its covalent structure, in view of the urea denaturation studies reported earlier (14). The last few residues at the *C*-terminal end of the chain (-Ala.Ser.Val) are, apparently, not obligatory in catalysis, as indicated by carboxypeptidase studies ((25) and unpublished results from Anfinsen and Richards). As shown by the experiments of Richards (12), a proteolytic cleavage with subtilisin does not affect activity. Activity is, however, completely abolished by oxidation of the disulfide bridges. The present results with pepsin introduce the following additional factors into the consideration of the chemical nature of the active center: (*a*) A *C*-terminal tetrapeptide, -Asp.Ala.Ser.Val, is removed during inactivation and its rate of appearance parallels the rate of inactivation. (*b*) The inactivated ribonuclease molecule has viscosity and sedimentation characteristics very similar to those of the native molecule, both before and after oxidation of disulfide bridges. (*c*) No new *N*-terminal amino acid residues can be detected in significant amounts in the inactive compound.

The combined available information would suggest that the aspartic acid residue, located four positions from the *C*-terminal residue, may be involved in the catalytic center of ribonuclease. However, since photooxidation studies (26) have shown that a histidine residue is probably also involved (suggested also by the studies of Kalnitsky and Rogers (27)), it is likely that loss of the aspartic acid-containing tetrapeptide represents only one of a number of possible simple inactivating procedures and that enzyme activity requires the organized participation of a constellation of amino acid residues. It should be emphasized here that 2 of the 4 histidine residues in ribonuclease are located in the last one-fifth of its polypeptide chain (20, 28).

According to thermodynamic considerations by Schellman (29, 30) and by Kauzmann (31), a non-cross-linked peptide chain of the length probably present at the *C*-terminal end of ribonuclease (about 15 residues) would not be likely to exist in an intrinsically stable, hydrogen-bonded form. The

earlier urea studies, therefore, are consistent with the present concepts of a *C*-terminal "tail" location for the active center, since the presence or absence of hydrogen-bonding in the rest of protein would be of no significance in catalysis. *Activity cannot, however, be due to a simple straight chain sequence of amino acids in view of the inactivating effect of disulfide bridge oxidation.*

SUMMARY

Although in a rather unsettled state at present, the hypothesis of the "denaturase" action of proteolytic enzymes (32) must be considered as one possible explanation for the inactivation observed during restricted pepsin digestion of ribonuclease. This is particularly true in view of the presence in ribonuclease of cross-linking bonds such as those between tyrosine hydroxyl groups and carboxyl groups suggested by Crammer and Neuberger (33) and more recently studied by Shugar (34), Harrington (35), and Tanford, Hauenstein, and Rands (36). (Indeed, in repeating Shugar's studies on the effect of pepsin digestion on the ultraviolet spectrum of ribonuclease, we have found that the rate of modification of one of these bonds parallels the rate of loss of enzyme activity.) The tyrosine hydroxyl-carboxyl interaction is structurally permissible by the amino acid arrangement (20, 28) at the *C*-terminal end of ribonuclease (although in this case, the bond would require unusual stability to urea in view of the "denaturation" studies (14) discussed above).

I would like to express my sincerest thanks to Professor K. Linderstrøm-Lang for his warm interest and hospitality during my all too short stay in his laboratory. I am grateful to Civilingeniør A. Johansen, Civilingeniør M. Ottesen, Dr. F. Richards, and Dr. W. F. Harrington for their advice and aid in these studies.

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THE OXIDATION OF MALIC ACID BY MICROCOCCUS LYSODEIKTICUS*

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The reaction catalyzed by mammalian malic dehydrogenase (1) is characterized by its dependence upon DPN,[1] inhibition by traces of OAA, and stimulation by carbonyl-binding compounds such as cyanide (2, 3). It was of interest, therefore, when Krampitz (4), studying oxidation of malate by whole or acetone-treated cells of *Micrococcus lysodeikticus*, found accumulation of OAA during the reaction. OAA, furthermore, failed to inhibit, and cyanide or semicarbazide did not stimulate the reaction. McManus (5) confirmed these observations and, in addition, could find no dependence of malate oxidation upon DPN or TPN.

The present investigation was initiated to study the manner in which malate is oxidized by *M. lysodeikticus*. Evidence has been obtained that at least two OAA-producing malic dehydrogenases are present in this microorganism, the first being DPN-dependent and the second not dependent upon this cofactor or TPN. Partial purification has permitted separation of these activities.

Methods and Materials

l-Malic Acid—*l*-Malic acid was obtained as the commercial product and was crystallized three times from acetone and benzene. Before use, it was dissolved in water and adjusted to pH 8 with NaOH.

OAA[2]—OAA was prepared from the commercial sodium salt of the diethyl ester (6, 7) and crystallized three times from acetone and benzene.

DPNH—DPNH was prepared from commercial DPN (Pabst) by hydro-

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[1] The following abbreviations are used in this report: diphosphopyridine nucleotide, DPN; reduced DPN, DPNH; flavin adenine dinucleotide, FAD; flavin mononucleotide, FMN; lactic dehydrogenase, LDH; malic dehydrogenase, MDH; oxalacetic acid, OAA; triphosphopyridine nucleotide, TPN; tris(hydroxymethyl)-aminomethane, Tris; sonic supernatant solution, SPS; R. Q., respiratory quotient; Am, ammonium sulfate.

[2] The author is indebted to Dr. M. F. Utter for a generous supply of this material.

sulfite reduction at 100° (8), followed by the separation of the salts by ethanol fractionation (9).

The following commercial chemicals were used: TPN, FAD, FMN (Sigma); DPN (Pabst); sodium pyruvate (Nutritional Biochemicals Corporation).

Lysozyme—This enzyme was obtained crystalline from the Armour Laboratories.

Analytical Determinations—Protein was determined by the biuret reaction with crystalline bovine serum albumin (Armour) as the reference standard. For determination of OAA, the method of manometric decarboxylation with Al^{+++} was employed (10).

Growth of M. lysodeikticus—An inoculum of *M. lysodeikticus* was prepared by transferring a loopful of cells which had been maintained on nutrient agar slants to 10 ml. of medium containing 1 per cent glucose, 0.4 per cent yeast extract, and 0.4 per cent Bacto-peptone. After shaking for 24 hours at 37°, the inoculum was added to 1 liter of the above medium in a 4 liter flask and swirled vigorously on a reciprocal shaker for 24 hours at 37°. The cells were harvested in a Sharples supercentrifuge, suspended in distilled H_2O, shaken for 1 hour, and recentrifuged. 5 to 7 gm. wet weight of cells from the Sharples supercentrifuge were collected per liter of growth medium.

Lyzing of Bacterial Cells—20 to 25 gm. of cells (wet weight) were suspended in 500 ml. of 0.01 M sodium phosphate buffer, pH 6.8, in 0.17 M NaCl. 25 mg. of lysozyme were added, and the mixture was gently agitated at 37° for 1 hour. The resulting lyzed preparation, somewhat gelatinous in consistency, was denoted *whole lyzed.*

Results

Manometric Assay of Malic Dehydrogenase—In early studies, oxygen consumption was utilized as a measure of enzymatic activity. However, following sonic treatment (below), oxygen consumption failed to yield a proper index of enzymatic activity, perhaps owing to loss or inactivation of accessory electron carriers to oxygen. This was apparent when the ferricyanide-acceptor assay of Quastel and Wheatley (11) was utilized in parallel experiments to test identical fractions (Table I). Although the actual amounts of malate oxidized (determined from gas exchange) in each system are not easily related, the ratios of activity before and following purification by each method should have been similar if no loss of cofactors occurred. This was not the case: An apparent loss of activity of 92 per cent was found with O_2 as acceptor, but only 52 per cent with the ferricyanide-acceptor system. Hence, the latter technique was employed in the standard assay in subsequent studies.

Contrary to the observation of Krampitz (4), cyanide was found to stim-

ulate malate oxidation in the presence of ferricyanide (Fig. 1) and, consequently, was included in the assay system. It should be noted, however, that a considerable reaction still occurred when this agent was omitted.

TABLE I

Activity of Successive Enzyme Fractions when Assayed with Both Oxygen and Ferricyanide As Electron Acceptor

Fraction	Malate oxidized*	
	O_2† acceptor	$Fe(CN)_6$‡ acceptor
Whole lyzed	51.8	31.7
Sonically treated (SPS)	3.9	15.1

* Micromoles per ml. of enzyme in 20 minutes.

† Warburg cups contained 0.06 M Tris buffer, pH 8.2, 0.06 M malate, and enzyme in a final volume of 2.0 ml. 0.3 ml. of 20 per cent KOH in the center well. Gassed with O_2 and temperature equilibrated for 5 minutes.

‡ Standard assay system (see the text) except that cyanide was omitted.

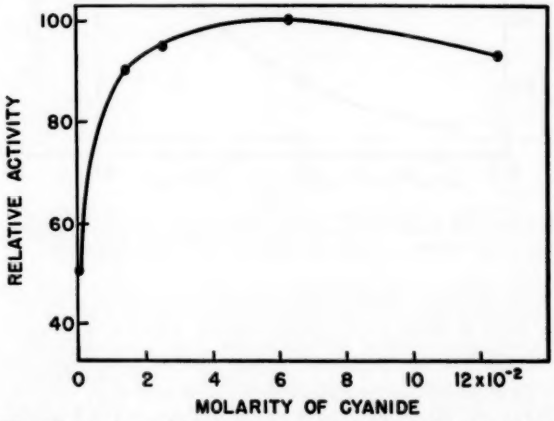

FIG. 1. The effect of cyanide on enzymatic activity with ferricyanide-acceptor system. Relative activity of 100 equals 1126 manometric units per ml. Fraction SPS.

The concentrations of cyanide shown in Fig. 1 are somewhat less than indicated, since an indeterminate amount of HCN was lost during the gassing procedure.

The pH-activity curve for MDH assay is presented in Fig. 2. The pH selected for the standard system was 8.2. The standard assay system,

conducted in Warburg flasks, final volume 2 ml., contained 0.13 M $NaHCO_3$, 0.03 M $K_3Fe(CN)_6$, and 0.05 M NaCN (pH 8.2). Following gassing with 5 per cent CO_2-95 per cent N_2 for 5 minutes and temperature equilibration for 5 minutes, 30 μmoles of malate were tipped in from the side arm and readings were taken after an additional 5 minutes. The temperature was 30°. 1 *manometric unit* equaled 0.001 μmole of malate oxidized per minute (calculated from the evolution of CO_2) over a 20 minute period.

Spectrophotometric Assay of Malic Dehydrogenase—All pertinent fractions were assayed also by measurement of the rate of reoxidation of DPNH

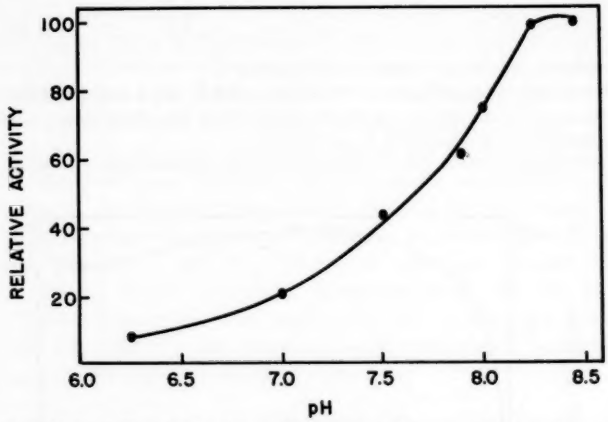

FIG. 2. Activity-pH relationship with ferricyanide-acceptor system. Relative activity of 100 equals 1210 manometric units per ml. Fraction SPS.

at 340 mμ following the addition of 6 μmoles of OAA to a cuvette containing enzyme, 0.04 M sodium phosphate buffer, pH 7.4, and approximately 0.2 μmole of DPNH at 30° (final volume 3.0 ml.). 1 *spectrophotometric unit* of activity equaled the reoxidation of 0.001 μmole of DPNH (or reduction of OAA) in the 30 to 90 second period following OAA addition.

Each fraction studied in this manner was assayed separately for LDH by use of pyruvate as substrate under identical conditions, since the presence of this enzyme would yield high values for DPNH reoxidation by MDH, if OAA should partially decompose to pyruvate.

Sonic Treatment of Residue after Lysis—Because studies on MDH were not previously performed on other than whole or lyzed cells, it was deemed advisable to obtain purer preparations. The whole lyzed fraction (above) was centrifuged for 30 minutes at 78,400 × *g* in a Spinco preparatory cen-

trifuge. The clear, yellow supernatant solution, which contained no malic dehydrogenase activity, was discarded and the gelatinous pellet was suspended in 100 ml. of 0.05 M NaCl with the use of a loose fitting Potter-Elvehjem homogenizer. 25 ml. aliquots were then subjected to 9000 cycle vibrations at full power for 45 minutes in a Raytheon 50 watt sonic oscillator and the sonic suspension was centrifuged at 78,400 × *g* for 1 hour. The clear, yellow supernatant solution was suctioned off, and the dark, brown pellet was discarded. The amount of ferricyanide-reacting activity found in the SPS fraction depended on the duration of sonic treatment of the whole lyzed fraction as shown in Fig. 3. After 45 minutes, inactivation was seen.

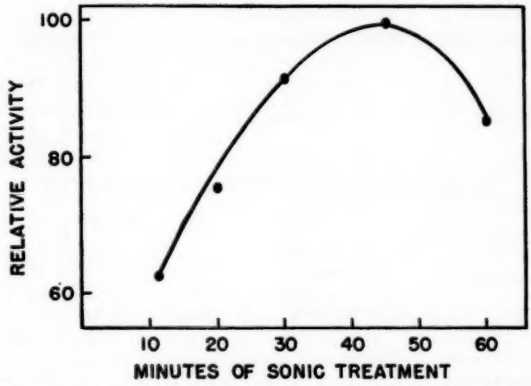

FIG. 3. The effect of sonic disruption on solubilization of MDH. Relative activity of 100 equals 1176 manometric units per ml.

Although sonic disruption was most effective for the liberation of enzyme, a small amount of activity could be released by glass grinding whole cells. However, freezing-thawing or extraction of acetone powders of lyzed cells failed to release activity.

Ammonium Sulfate Fractionation—The SPS was made 0.03 M with Na_2HPO_4 and 52 per cent ammonium sulfate (weight per volume) was added dropwise with stirring at 4° to give an ammonium sulfate concentration of 20 per cent (assuming additive volumes), during which procedure a precipitate was formed. Stirring was continued for an additional 30 minutes. The precipitate was collected by centrifugation (35,000 × *g*, 15 minutes) and dissolved in 0.05 M NaCl. The supernatant solution was then treated as above to bring the concentration of ammonium sulfate to 30 per cent. The precipitate (Am 20–30) was centrifuged and dissolved in 0.05 M sodium chloride. The supernatant solution was brought to a concentration of 40

per cent ammonium sulfate as above and the resultant precipitate was centrifuged and collected for analysis (Am 30–40).

Table II presents a summary of the yields of enzymatic activities as assayed by both spectrophotometric and manometric assays. Although variations in activity occurred in different preparations, the values shown are typical. These results demonstrated that a marked separation of activities could be obtained. The ferricyanide-reacting enzyme appeared essentially in the 0–20 fraction, while the DPN-reacting activity appeared primarily in the 30–40 fraction. The Am 20–30 fraction contained a mixture of both enzymes. Relative to the whole lyzed fraction, the ferricyanide-reacting enzyme was purified 5-fold. The DPN-reacting enzyme was purified to a similar degree when compared to the SPS fraction. It was of interest to note that the whole lyzed preparation failed to show activity with DPN. After sonic treatment, however, a large amount of spectrophotometric activity was observable. It would appear that the enzyme contained within this fraction was inaccessible to the substrate, possibly because of its gelatinous consistency. This finding might also explain the inability of McManus (5) to observe a reaction with DPN in her studies.

TABLE II

Summary of Malic Dehydrogenase Purification Procedure

Fraction	Protein	Manometric		Spectrophotometric	
		Units per mg. protein	Total units	Units per mg. protein	Total units
	mg.				
Whole lyzed	2760	122	337,000	0	0
Sonic suspension	792	*	*	30	23,700
SPS	463	337	156,000	34	15,700
Am 0–20	186	602	112,000	4	740
" 20–30	84	409	34,400	42	3,500
" 30–40	60	44	2,600	167	10,000

* Not assayed.

An alternative explanation, however, which had to be explored was that the sonic treatment disrupted a natural (non-DPN) enzyme-acceptor complex and created an artifactitious enzyme which could then react with DPN. To test this, the Am 0–20 fraction was subjected to additional sonic treatment for 20 minutes and then assayed (Table III). It can be seen that sonic disruption failed to increase the ability of this fraction to react with DPN; rather the small amount of DPN activity was further diminished. Conversely, sonic treatment of the Am 30–40 fraction failed to create activity as measured by manometric assay.

Stability—All fractions of enzymes appeared to be stable in solution at 3–4°, with only slight loss of activity after 4 weeks. Furthermore, freshly grown cells have been lyophilized and maintained *in vacuo* for 2 years, after which the above fractionation procedure was carried out. Loss in activity, assayed manometrically, amounted to about 40 per cent.

Dialysis—When SPS was dialyzed for 48 hours against distilled H_2O, the DPN-dependent fraction appeared to be considerably less stable than

TABLE III

Effect of Sonic Treatment on Am 0–20 Fraction

Treatment	Manometric	Spectrophotometric
	Units per mg. protein	Units per mg. protein
Untreated	540	5
Sonically treated	493	0

TABLE IV

Effect of Dialysis of SPS upon Enzymatic Activity

Fraction	Activity	
	Manometric	Spectrophotometric
	units per ml.	*units per ml.*
Aged control	380	133
Dialyzed	98	5
Ratio, $\frac{\text{aged}}{\text{dialyzed}}$	3.9	27

the non-DPN enzyme (Table IV). These data confirm the existence of two enzymes in the preparation.

Theoretical Gas Exchange and R. Q.—When oxygen uptake and CO_2 evolution were determined in crude preparations (whole lyzed), values were obtained which were close to theoretical for the reaction sequence:

$$\text{Malate} + \tfrac{1}{2}O_2 \rightarrow \text{OAA} \rightarrow \text{pyruvate} + CO_2 \ (\text{R. Q.}_{\text{theory}} = 2.00)$$

Thus, in a typical experiment 27.9 μmoles of CO_2 were evolved and 13.7 μmoles of O_2 were consumed when 30.0 μmoles of malate were used. Similarly, in the case of the ferricyanide assay system, final CO_2 evolution approximated the theoretical if the amount of malate was decreased so that excess ferricyanide was present (Table V, Am 0–20). It is of significance in these experiments that no enzyme fraction following sonic treatment

exhibited significant lactic dehydrogenase activity either manometrically or spectrophotometrically.

Formation of OAA—OAA readily accumulated during the oxidation of malate, as presented in Table V. In these studies, the manometric assay system was employed, with the exception that cyanide was omitted. The reactions were stopped within 20 minutes to decrease loss of OAA through spontaneous or enzymatic decarboxylation. As purification proceeded, the percentage of OAA formed increased until with the Am 0–20 fraction almost complete conversion to OAA was observed. These data suggest the progressive loss of OAA decarboxylase (present in large amount in *M. lysodeikticus* (12)) during purification.

Effect of DPN—The effect of DPN on the Am 0–20 and 30–40 fractions

TABLE V

Accumulation of OAA during Malate Oxidation

Preparation	Malate oxidized*	OAA found	Conversion to OAA
	μmoles	*μmoles*	*per cent*
Whole lyzed†	16.3	1.7	10
SPS†	4.7	3.2	68
Am 0–20‡	22.7	22.1	97

* Estimated from CO_2 evolution.

† Standard manometric assay system except cyanide omitted.

‡ Same as for SPS but 24.0 μmoles of malate added. Reaction allowed to go to completion.

differed considerably. As indicated in Table II, the former fraction retained little activity when assayed spectrophotometrically. On the other hand, the Am 30–40 fraction reacted with DPN in a manner quite similar to that of mammalian malic dehydrogenase. Fig. 4 illustrates both the back reaction (OAA → malate) (Curves a and b) and the forward reaction (malate → OAA) (Curves c and d). To emphasize the forward reaction, a concentration of malate 10 times that of OAA was used and the amounts of DPN and enzyme were increased. Even so, addition of small amounts of OAA completely reversed the equilibrium. The use of hydrazine as the trapping agent (Curve d) produced a slow forward reaction which is typical for animal malic dehydrogenase.

Several manometric experiments were performed to clarify the need of the Am 0–20 and Am 30–40 fractions for DPN (Table VI). In no case was DPN demonstrated as essential for the Am 0–20 fraction. Likewise, after shaking with Norit, a treatment which can remove by adsorption complex-formed DPN (13), or following dialysis, DPN failed to stimulate the oxida-

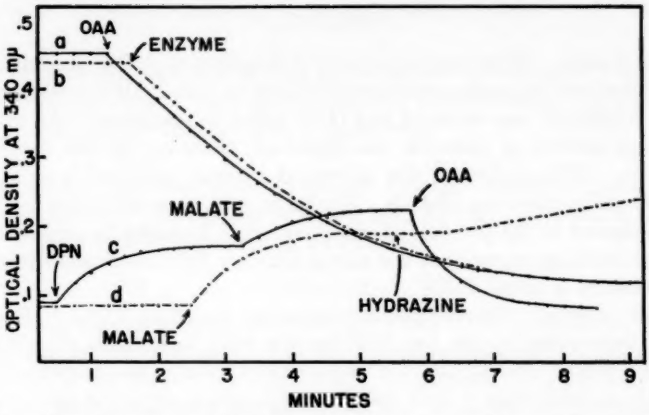

FIG. 4. Optical tests with MDH of fraction Am 30–40. All cuvettes contained 120 μmoles of sodium phosphate buffer, pH 7.4. Curve a, 0.16 μmole of DPNH, MDH (0.027 mg. of protein); 6 μmoles of OAA added at arrow. Curve b, same as Curve a, except reaction started by adding MDH at arrow. Curve c, MDH (0.087 mg. of protein), 60 μmoles of sodium malate; reaction started by adding 1.0 μmole of DPN; an additional 60 μmoles of malate added at arrow; 3 μmoles of OAA added at final arrow. Curve d, MDH (0.087 mg. of protein), 1.0 μmole of DPN; 60 μmoles of sodium malate and 0.32 mmole of hydrazine sulfate added when indicated. Silica cells, d = 1.0 cm.; temperature 30°. Curves corrected for optical density of substances added and for dilution. Data transcribed from per cent transmission curves obtained with model 5800 energy recording adapter for Beckman DU spectrophotometer and Bristol Dynamaster strip chart recorder.

TABLE VI

*Effect of DPN on Active Fractions**

Fraction	Treatment	Manometric activity		
		No addition	+DPN†	Activation by DPN
		units per ml.	*units per ml.*	*per cent*
Am 0–20	Untreated	583	574	0
	Dialyzed 24 hrs.‡	293	293	0
	+0.45 M nicotinamide	297	298	0
	Norit-treated§	1380	1240	0
Am 30–40	Untreated	526	635	21
	Dialyzed 18 hrs.‡	252	351	39
	+0.45 M nicotinamide	160	271	69
SPS	Untreated	1110	1200	8
	Dialyzed 48 hrs.‡	781	870	11

* All fractions from same batch except Norit-treated.

† 1.36 μmoles of DPN per ml.

‡ *Versus* 0.005 M sodium phosphate buffer, pH 8.0.

§ Procedure for adsorption is that of Taylor *et al.* (13).

tion of malate. When the test system of Feigelson *et al.* (14), in which high concentrations of nicotinamide are employed to inhibit DPN enzymes, was used, inhibition was observed but DPN failed to reactivate. An entirely different pattern of behavior was observed, however, for the Am 30–40 fraction. DPN activated the untreated fraction and had a somewhat greater effect following dialysis. Similarly, a large reactivation by DPN was observed in the presence of nicotinamide. As might be expected, the malate-oxidizing capacity of the parent fraction, SPS, increased slightly in the presence of added DPN.

Other Cofactors—Several possible cofactors, including TPN, FMN, and FAD, were added to the Am 0–20 fraction both before and after dialysis without effect. In addition, the various ammonium sulfate fractions failed to activate each other in both assay systems, which suggested that no activating factors were removed from the enzymes during fractionation.

DISCUSSION

The data support the existence of two separate enzymes for the oxidation of malic acid to OAA in *M. lysodeikticus*. The MDH of the Am 30–40 fraction resembles that of pig heart purified by Straub (1) in its requirement for DPN and in its equilibria established when observed spectrophotometrically (2). The second enzyme (Am 0–20 fraction) does not require DPN (or TPN) for its reaction. Similarly, cyanide stimulates oxidation of malate but is not essential for complete utilization of substrate which is the case with pig heart malic dehydrogenase. A non-DPN malic dehydrogenase, recently reported by Scholefield (15) to be present in pigeon liver, might be similar to the one found here.

That OAA is the product of the oxidation of malate by the Am 0–20 fraction is evident from theoretical oxygen consumption, R. Q., accumulation of aluminum-decarboxylatable material following oxidation and stimulation of the reaction by cyanide. However, this conclusion is not unequivocal, since in this method of identification of OAA by β-decarboxylation with Al^{+++} it might not be possible to distinguish between OAA and a labile precursor. Furthermore, evidence exists which suggests the existence of such a labile substance. Thus, Krampitz suggested that the product of malate oxidation in *M. lysodeikticus* was a "physiological oxalacetic acid" (4, 16). This postulate was based on the observation that, when authentic OAA was added to acetone-treated *M. lysodeikticus*, complete disappearance was observed, whereas OAA accumulated when malate was utilized as substrate. Furthermore, this "physiological OAA" was decarboxylated at a rate slower than authentic OAA, a finding which was confirmed by McManus (5). Although the present study sheds no light upon the possible formation of "physiological OAA," the existence of a

novel enzyme for malate oxidation points up the necessity of a detailed study of this reaction. Such an investigation which includes further purification and cofactor requirements is under way.

The author is indebted to Dr. Merton F. Utter for his advice and help in this investigation. The author wishes to acknowledge the technical assistance rendered by Mr. Raymond R. Newman.

SUMMARY

Two enzymes have been observed in *Micrococcus lysodeikticus* which oxidize malate to oxalacetate. The first resembles mammalian malic dehydrogenase in its dependence upon DPN, stimulation by hydrazine, and equilibria established between substrates and products. The second enzyme is stimulated by cyanide but shows no dependence upon DPN or TPN and fails to react significantly with DPN when observed spectrophotometrically. Partial purification has permitted separation of the two malic dehydrogenase activities.

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METABOLISM OF THYMINE (METHYL-C^{14} OR -2-C^{14}) BY RAT LIVER IN VITRO*

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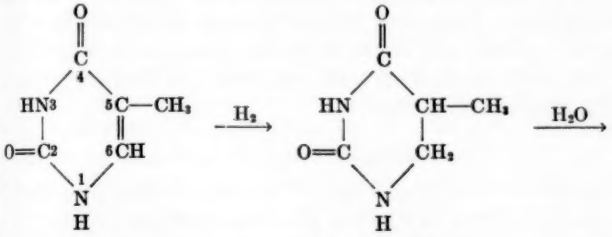
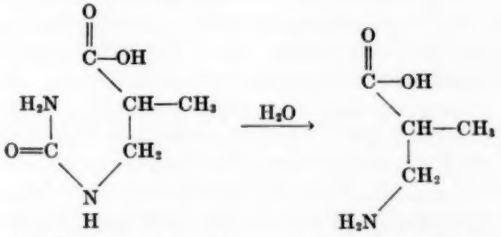
Previous investigations employing rats, microorganisms, and surviving liver from several species (1–7) indicated that β-aminoisobutyric acid[1] can be formed metabolically through the following reactions:





DHT and BUIB were found to be extensively converted to BAIB by rat liver (3, 5, 7), with little doubt that a real precursor-product relationship

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[1] The abbreviations used in this paper are BAIB, β-aminoisobutyric acid; BUIB, β-ureidoisobutyric acid; DHT, dihydrothymine; BAIB amide, β-aminoisobutyramide; HMU, 5-hydroxymethyluracil; HMC, 5-hydroxymethylcytosine.

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was involved in these cases, but the yield of β-amino acid from thymine itself has always been low in the mammalian systems tested, and confirmation of the over-all reaction by use of suitably labeled radiothymine appeared desirable. The relatively easily labeled ureide carbon of thymine would be lost in the reactions as written above; thus the methyl carbon was chosen for labeling on the basis that it would presumably appear in the β-amino acid and might conceivably reenter the "pool" of single carbon units to some extent or present some other special type of metabolism.

A preliminary report of these experiments (8) described chromatographic tests which confirmed the expectation that rat liver slices would convert thymine (methyl-C^{14}), but not thymine-2-C^{14}, to radioactive BAIB. The thymine-2-C^{14} yielded radioactive urea and CO_2, as was anticipated from previous reports on mammalian catabolism of non-isotopic thymine (9), thymine-1,3-N^{15} (10), and thymine-2-C^{14} (11), and both types of labeled thymine showed a number of radioactive metabolites which were not immediately identifiable. These now appear to include intermediate reductive products (*e.g.* DHT and BUIB), oxidative products (*e.g.* 5-hydroxymethyluracil and uracil-5-carboxylic acid), and some less obviously related compounds (*e.g.* alanine and glucose).

EXPERIMENTAL

Thymine (methyl-C^{14}) was synthesized (8, 12) from sodium propionate-3-C^{14}, with a specific activity of 0.45 mc. per mmole, supplied by the Oak Ridge National Laboratory. Thymine-2-C^{14}, with a specific activity of 3.0 mc. per mmole, was obtained from the Isotopes Specialties Company, Inc. 5-Hydroxymethyluracil was prepared by deamination of 5-hydroxymethylcytosine[2] with barium nitrite and HCl. 5-Methylbarbituric acid was obtained by the procedure of Gerngross (13), and thymine glycol (5,6-dihydroxythymine) by a slight modification of the method of Baudisch and Davidson (14). Methylmalonic acid was obtained by hydrolysis of the ester, which was prepared according to Cox and McElvain (15).

Incubations were carried out with 500 γ of radiothymine, a 50 mg. slice of rat liver, and 500 μl. of Krebs-Ringer phosphate buffer in a 5 ml. beaker which was supported in a 10 ml. beaker, which, in turn, was placed in a 20 ml. beaker containing 3 ml. of 0.15 N NaOH. The top of the outer beaker was sealed with Parafilm, the unit was placed in a Dubnoff metabolic shaker at 37°, and after 5 hours the incubation medium was deproteinized by addition of 4 volumes of absolute ethanol. A 20 μl. aliquot of the supernatant fluid was dried as a small spot near one corner of a sheet of filter paper (20 × 21 cm.), which was formed into a cylinder and placed in a

[2] We are indebted to Dr. Charles Miller of Sharp and Dohme, and to Dr. A. Deutsch of the California Foundation for Biochemical Research for their gifts of HMC.

half gallon fruit jar at 28° for ascending development as a two-dimensional chromatogram, an ammoniacal system (Solvent 1, Table I) being used first and then a neutral mixture (Solvent 2, Table I). The chromatograms were pressed against x-ray film, usually for 1 to 3 weeks, pinholes having been pierced to facilitate subsequent realignment of the radioautographs.

The various radioactive spots were cut from replicate initial chromatograms prepared as above, eluted with ethanol or ethanol-water mixtures, and rechromatographed in one or more additional pairs of solvents to obtain further purification and a set of at least four R_F values for each radioactive spot. In several instances all of the values so obtained corresponded closely with a similarly determined set of R_F values of a known compound, thus suggesting the identity of some of the radioactive metabolites. Such a radioactive spot was then eluted from the second set of chromatograms, and chromatographically similar compounds were added to a portion of the eluate to serve as non-radioactive markers. The mixture was then rechromatographed, by using a pair of solvents which appeared most likely to be useful for detecting any differences between the radioactive and the non-radioactive spots. Radioautographic exposures were made, as long as 2 months being required for adequate registration of isolated samples from some of the minor radioactive spots. The position, shape, and internal density gradients of the radioautographic spots were then critically compared with the same properties of the spots representing the added non-radioactive markers, as visualized on the chromatogram by examination in ultraviolet light or by appropriate color reactions. When an isolated radioactive compound appeared to coincide with one of the added markers, the pair was usually retested in additional solvent systems, and an effort was made to make all feasible comparisons with other compounds which might be expected to show similar chromatographic properties and to be potential thymine metabolites.

Color-developing reagents were sprayed and dried very carefully in order to avoid displacing or distorting colored spots, and a long series of comparisons between a non-radioactive marker and a moderately to highly radioactive compound rarely left any doubt regarding their identity or non-identity.

Identification of the metabolites was the primary aim of this investigation, and comparison of the relative amounts of radioactivity associated with various spots was ordinarily limited to the rough estimates obtainable by visual inspection of the radioautographs.

Results

An initial chromatogram of a deproteinized incubation medium, prepared as outlined above, is represented by the radioautograph reproduced in Fig.

1. The residual substrate, thymine (methyl-C^{14}), was the most intensely radioactive spot (*T*), and some of the spots (*A*) were apparently chemical or physical artifacts, since they reappeared when the thymine was eluted, concentrated, and rechromatographed in the same solvent systems. Thymine glycol took a chromatographic position in the upper artifact spot on this initial type of chromatogram and ran with, or near, a weakly radioactive component of that spot in some of the other solvents. Further studies would be required to determine whether radioactive thymine glycol was actually produced in these experiments and, if so, whether it was formed spontaneously, biologically, or in both ways. Preliminary tests also sug-

FIG. 1. Radioautograph from a chromatogram of liver incubation medium. +, starting point of chromatogram; *T*, residual substrate, thymine (methyl-C^{14}); *A*, chemical or physical artifacts; *B*, BAIB plus alanine; *G*, glucose; *H*, HMU.

gest that the unidentified compound near the lower left edge of the thymine spot may be a hydroxymethyluracil nucleoside, but confirmatory data are needed. Urea was present on the chromatogram to the left of the thymine, and the absence of a corresponding radioautographic spot indicates that there was little or no conversion of the labeled methyl group to urea. (There was also little formation of $C^{14}O_2$ from the methyl carbon.) Each of the spots in Fig. 1 not labeled as an artifact or substrate presumably represents one or more radioactive metabolites, or subsequent breakdown products thereof, and notations indicate the positions of compounds which have been chromatographically identified.

The data obtained from the various types of chromatograms and the two types of labeled thymine are collected in Table I, which shows R_f values of unlabeled marker compounds and of the radioactive spots with

which some of them appear to be identical. The relative intensities of the radioactive spots are indicated by a rough scale which reflects the higher initial specific activity of the thymine-2-C^{14} and labels as doubtful (±) those radioactive spots which were too weak to be detected consistently or identified adequately. In many cases, no metabolite with detectable radioactivity showed the chromatographic properties of the unlabeled compound in all of the solvent systems tested, but this does not necessarily rule out the possibility that the compound tested might be an important, but perhaps transitory, intermediate in thymine metabolism. Such a situation presumably was involved in the failure to obtain unequivocal identification of DHT and BUIB as radioactive metabolites of thymine (methyl-C^{14}), for these products carried comparatively little radioactivity even when the highly radioactive thymine-2-C^{14} was used as the substrate.

DISCUSSION

Interpretation of previous data on the apparent precursor-product relationship between thymine and BAIB required consideration of the alternative possibility that thymine might serve merely as an indirect stimulus to the production or release of BAIB by liver tissue (3). This now appears to have been ruled out adequately by the identification of BAIB as a radioactive metabolite of thymine (methyl-C^{14}).

Detection of radioactive DHT and BUIB in the experiments with thymine-2-C^{14} similarly constituted the first direct evidence for the production of these compounds as intermediates during mammalian catabolism of thymine. The occurrence of DHT and BUIB under such conditions had previously seemed probable, however, (*a*) because liver was found to have active enzyme systems for their hydrolysis (3, 5, 7), (*b*) because they accumulated in colorimetrically detectable concentrations during bacterial metabolism of thymine (4), and (*c*) because they could be isolated as intermediates when BAIB was prepared in stepwise fashion by catalytic hydrogenation of thymine to DHT, mild alkaline hydrolysis of the DHT to BUIB (4, 16), and acid hydrolysis of the BUIB. At least the first two steps of the above reaction sequence appear to be biologically reversible (4–7), suggesting a possible anabolic significance for this reductive pathway of thymine metabolism.

Detection of the easily metabolizable BUIB in these studies and failure to detect BAIB amide, which seems relatively stable in mammalian systems (5–7), add to the evidence that biological cleavage of the reduced pyrimidine ring occurs primarily, if not exclusively, between positions 3 and 4 (4–7, 17, 18). This view is consistent with a variety of data which indicate that the urea moiety of pyrimidines tends to be fragmented during catabolism (2, 11, 19, 20), but it does not rule out the existence of competing

TABLE I

Data on Compounds Investigated As Possible Metabolites of Thymine

An R_F value printed in bold-faced type indicates that the corresponding solvent was employed for a direct comparison of the radioactive and non-radioactive compound on the same chromatogram; italic type indicates close agreement of R_F values obtained from separate chromatograms; R_F values determined for the unlabeled compounds only are printed in light-faced type.

Non-radioactive marker compound	Associated radioactivity from thymine*		R_F values (× 100) in solvent No.†							
	(Methyl-C^{14})	-2-C^{14}	1	2	3	4	5	6	7	8
Thymine	+++	++++	**55**	**76**	**68**	**64**	**58**	30	80	**33**
HMU	++	+++	**30**	**53**	**47**	**37**	**32**	10	**65**	6
Glucose	++	±	**40**	**37**	**32**	**18**	**9**	8	65	0
BAIB	++	−	***33***	**34**	**65**	**66**	**13**	9		0
Alanine	+	±	***30***	***35***	**49**	**57**	**3**	6	59	0
Urea	±	+++	**57**	**49**	**59**	**58**	**28**	26	68	26
DHT	±	++	***78***	***71***	69	**67**	**58**	**48**	81	48
BUIB	±	++	**34**	***32***	**75**	**78**	**68**	5	95	47
Uracil-5-carboxylic acid	±	+	***8***	**20**	**47**	**43**	43	0	**57**	25
Thymine glycol	±	±	***79***	***76***	78	77	78	51	82	61
BAIB amide	−	−	70	40	57	45	6	41		0
5-Methylbarbituric acid	−	−	35	26	51	60	26	8	70	54
5-Methylcytosine	−	−	53	56	61	52	14	32	53	0
HMC	−	−	40	43	49	38	10	15	45	0
Methylmalonic acid	−	−	17		81	90	90	0		85
Thymidine	−	−	58	77	68	64	60	27	89	19
5,6-Dihydrothymidine	−	−	70	63	62	50	37	32	77	17
Thymidylic acid	−	−	5	28	36	25	0	0	89	0

* + = presence of, ± = doubt as to presence or identity of, and − = absence of a radioactive counterpart in the radiothymine incubation media. Relative intensity is indicated by the number of plus signs.

† Composition of solvent systems, by volume: Solvent 1, *tert*-butyl alcohol, methylethyl ketone, water, ammonium hydroxide (40:30:20:10); Solvent 2, upper phase from a mixture of water, *sec*-butyl alcohol, *tert*-butyl alcohol (47.5:42.5:8.5); Solvent 3, *n*-butyl alcohol, glacial acetic acid, water (50:25:25); Solvent 4, *tert*-butyl alcohol, methylethyl ketone, formic acid, water (40:30:15:15); Solvent 5, *tert*-butyl alcohol, methylethyl ketone, water, formic acid (44:44:11:0.264); Solvent 6, upper phase from a mixture of *n*-butyl alcohol, water, and ammonium hydroxide (60:30:10); Solvent 7, isopropyl alcohol, water, concentrated HCl (65:18.4:16.6); and Solvent 8, upper phase from a mixture of ethyl acetate, water, formic acid (60:35:5). When a two-phase system was used, a 20 ml. beaker containing 15 ml. of the lower phase was placed in the bottom of the chromatographic jar.

mechanisms such as those involving removal of the urea moiety as an intact unit (9, 21–26). There appears, at present, to be little experimental basis for the suggestion (20) that removal of the ureide carbon constitutes the primary step in pyrimidine catabolism.

If the conversion of BUIB to BAIB involved an initial hydrolytic removal of the amide group representing position 3 of the original pyrimidine ring, it seems probable that the resulting *N*-substituted carbamic acid would have broken down to BAIB and CO_2 before or during analysis. It is also conceivable that positions 2 and 3 might be removed together as carbamic acid, and partially stabilized as a salt or ester. Both of the above possibilities were of special interest since some carbamate derivatives have been found to stimulate the development of lung tumors in strain A mice (27–29), and high BAIB excretion has been noted in a number of cases of human cancer (1). No radioactive carbamates have been identified in these studies, however, and negative results were obtained when urethan-sensitive mice were used to test for production of oncogenic carbamates during extensive biological conversion of dihydropyrimidines to β-amino acids (30).

Liver enzymes can transfer the amino group from BAIB to α-ketoglutarate or pyruvate,[3] but in thymine (methyl-C^{14}) experiments none of the radioactive spots were clearly identified as immediate or secondary products of BAIB deamination. The radioactive alanine and glucose could conceivably have been derived from thymine (methyl-C^{14}) either with or without BAIB as an intermediate, and speculation as to possible mechanisms for their production seems premature.

Study of the radioactive chromatograms has provided support for the earlier suggestion (2) that mammalian tissues might be provided with alternative pathways for thymine metabolism, with one route involving an initial reduction and the other an initial oxidation. There remains some question regarding mammalian oxidation of thymine to thymine glycol, as postulated by Cerecedo (9), and our radioautographs did not show 5-methylbarbituric acid, which was isolated by Hayaishi and Kornberg (23, 24) as an acid-labile microbiological oxidation product of thymine. The negative results are particularly inconclusive in the latter case, owing to the lability of the product, however, and in order to determine whether oxidations of this type occur in mammalian tissues it might be preferable to use labeled uracil and to search for barbituric acid, a relatively stable pyrimidine metabolite produced by bacterial oxidation (19, 23–26). The finding of HMU as one of the major radioactive derivatives of labeled thymine suggests that the methyl group of thymine may be the principal target for oxidative attack by liver tissue. In a preliminary report, Batt and Woods

[3] Unpublished experiments.

(31) suggested that HMU might be the initial compound formed during bacterial oxidation of thymine, but, in the light of more recent data, the reported characteristics of their compound are seen to correspond closely with those of 5-methylbarbituric acid, as pointed out by Hayaishi and Kornberg (24), and do not appear to match those of HMU (32). The closely related compound HMC, however, was isolated from T-even coliphages by Wyatt and Cohen (32, 33), and, although HMU was not detected in either the phage or the host, its deoxyriboside could be formed slowly from that of HMC by action of *Escherichia coli* deoxycytidine deaminase (34). The uracil-5-carboxylic acid chromatographically identified in the present studies would be a logical oxidation product of HMU, but the initial conversion of the methyl group of thymine to the relatively reactive hydroxymethyl group could conceivably open the way for its participation in a variety of anabolic reactions. HMU has been postulated as an intermediate in the formation of phage HMC (32, 34, 35), but in that system the hydroxymethyl group appears to be derived primarily from molecules other than thymine (34–36). A synthesis of isotopic HMU is under way as a step toward a study of its metabolism in mammalian systems.

SUMMARY

On incubation with rat liver, thymine (methyl-C^{14}) yielded a number of radioactive metabolites, some of which have been chromatographically identified as β-aminoisobutyric acid, 5-hydroxymethyluracil, alanine, and glucose. With a more highly radioactive preparation of thymine-2-C^{14}, the detectable radioactive products included 5-hydroxymethyluracil, urea, dihydrothymine, β-ureidoisobutyric acid, and uracil-5-carboxylic acid. A large amount of $C^{14}O_2$ was produced from the ureide carbon and only a small amount from the methyl group. The data provide direct evidence for the reductive conversion of thymine to β-aminoisobutyric acid, for the occurrence of dihydrothymine and β-ureidoisobutyric acid as intermediates, and for the existence of an alternative metabolic pathway involving an initial oxidative attack on the methyl group of thymine.

Addendum (March 20, 1956)—An additional metabolite of radiothymine, shown near the lower left edge of the thymine spot in Fig. 1, has now been chromatographically identified as thymine riboside (5-methyluridine). The non-radioactive marker was prepared by treatment of uridine with formaldehyde (37), followed by hydrogenation of the resulting 5-hydroxymethyluridine to yield 5-methyluridine. The radioactive and non-radioactive products showed identical behavior on direct chromatographic comparison in Solvents 1 through 7 of Table I (R_F values × 100 were 44, 61, 56, 44, 37, 3, and 72, respectively), and both chromatographic and spectrophotometric studies indicated that the marker compound was identical with a sample of synthetic 1-D-ribofuranosyl-thymine (38) courteously provided by Dr. J. J. Fox and Dr. G. B. Brown.

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METABOLISM OF Δ^4-CHOLESTENONE-4-C^{14} IN THE RAT*

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Δ^4-Cholestenone has long been regarded as a key intermediate in the metabolism of cholesterol. Two rôles have been postulated for it: (*a*) Rosenheim and Webster proposed that Δ^4-cholestenone is an intermediate in the degradation of cholesterol to bile acids (1); (*b*) numerous investigators have considered it an intermediate in the conversion of cholesterol to the neutral fecal sterols, especially cholestanol (dihydrocholesterol) and coprostanol, and to tissue cholestanol (2, 3). The second scheme, which has recently been reviewed by Bloch (3), is supported by considerable evidence. Cholestenone has been isolated from feces (4), and evidence for its occurrence in certain tissues has been presented (5, 6). It is a well known product of microbial oxidation of cholesterol (7, 8). The conversion of cholestenone to cholestanol (9, 10) and to coprostanol (11) has been demonstrated. None the less, the recent demonstration of Rosenfeld *et al.* (12) that intestinal microorganisms can convert cholesterol to coprostanol without oxidation at C-3 indicates, at least, the existence of an alternative pathway between these two compounds.

The first part of the present communication deals with the fate of intravenously injected Δ^4-cholestenone-4-C^{14} in rats. In the second part, the conversion of cholestenone to cholestanol by liver homogenates is studied.

EXPERIMENTAL

C^{14}-Labeled Sterols—Δ^4-Cholestenone-4-C^{14} was synthesized as described by Turner (13). The product was isolated and purified on alumina columns, and the purity was established by melting point and paper chromatography (14).

Cholestanone-4-C^{14} was prepared by oxidation of cholestanol-4-C^{14} by the method of Bruce (15). The labeled cholestanol was prepared by Dr. W. G. Dauben, to whom our thanks are due. The cholestanone-4-C^{14} was isolated by alumina column chromatography as described below. Purity of the isolated material was established by recrystallization of the tetrahydrocarbazole derivative to constant specific activity (see below).

Unlabeled Sterols—Commercial cholestanol was recrystallized several

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† Predoctoral Fellow of the Life Insurance Medical Research Fund.

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times before use. Δ^4-Cholestenone and cholestanone were synthesized as described above.

Bile Acids—Commercial cholic and deoxycholic acids were recrystallized before use. Chenodeoxycholic acid was obtained by alkaline hydrolysis of taurochenodeoxycholic acid isolated from goose bile, as described previously (16). Recrystallization from ethyl acetate yielded chromatographically pure material, m.p. 137–139°.

Emulsification of Sterols—Labeled sterols were emulsified in saline with Tween 20 as described previously (17). Since Tween 20 is apparently toxic both to intact rats and to liver homogenates, the quantity used must not exceed 1 drop per mg. of sterol.

In Vitro Procedures—Liver homogenates were prepared as described by Bucher (18). Unless otherwise stated, 0.1 mg. of the C^{14} sterol, emulsified in 0.1 ml. of saline with Tween 20, was incubated with 2 ml. of homogenate, 0.2 ml. of 8×10^{-3} M adenosine triphosphate (ATP), and 0.2 ml. of 1.5×10^{-2} M diphosphopyridine nucleotide (DPN) for 1 hour, at 37°, with continuous shaking, in an atmosphere of 95 per cent O_2-5 per cent CO_2. At the end of the incubation period the mixture was hydrolyzed on a steam bath by addition of alkali, and the neutral sterols were isolated by solvent extraction as described below.

Methods

Small aliquots of bile were mounted directly on aluminum planchets and counted with a Q gas flow counter to an accuracy of ±5 per cent. Feces were exhaustively extracted with ethanol in a Soxhlet apparatus, and a small aliquot of the extract was used for C^{14} determination. Tissue C^{14} determinations were made after solvent fractionation.

Solvent fractionation was carried out on hydrolyzed bile and tissues, as described earlier (19).[1]

Chromatographic Separation of Bile Acids—Whole bile samples were chromatographed on filter paper, as described previously (20). The solvent used was collidine-H_2O (100:35 volume per volume) in an ammonia atmosphere. Larger samples were chromatographed on hydrophobic Super-Cel columns, as described by Bergström and Sjövall (21).

Chromatographic Analysis of Neutral Sterols—Neutral sterols were separated chromatographically on alumina columns. The sterols were applied to the column in hexane and were eluted with gradually increasing concentrations of ethyl ether in hexane. The effluent was collected in fractions and the C^{14} content of these fractions determined. The ratio of alumina to sterol was 100:1 or greater. Since commercial alumina (Merck) is not always reproducible, each batch was standardized before use.

[1] When total C^{14} alone was desired, the hydrolysate was acidified, and the total C^{14} was extracted with ethyl ether.

Identification of C^{14} Compounds—Preliminary identification of C^{14} compounds was carried out by column chromatography. To establish the identity of any given C^{14} substance, carrier was added and the initial specific activity determined. The mixture was then recrystallized several times from three different solvents, and the specific activity again determined. One or more derivatives were then prepared, again recrystallized several times, and the final specific activities determined. Constancy of the specific activity was interpreted as evidence of identity of the C^{14} substance with the carrier. The importance of preparing derivatives should be emphasized. Since sterols tend to coprecipitate with each other, recrystallization to constant specific activity without conversion to a derivative does not constitute sufficient evidence of identity.

TABLE I

Distribution of C^{14} of Injected Δ^4-Cholestenone-4-C^{14} in Organs and Excreta of Intact Rats

Each rat received intravenously 1 mg. of the labeled sterol emulsified in saline with Tween 20.

Tissue or site	Per cent of injected C^{14} recovered		
	In neutral fraction	In acidic fraction	Total
Feces	17	33	50.0
Liver	5.4	2.8	8.2
Intestine* + contents	5.4	5.4	10.8
Lungs			3.6
Spleen			1.6
Kidneys + adrenals			2.8
Carcass			17.0
Total			94.0

* Small and large intestine.

Results

Fate of Δ^4-Cholestenone-4-C^{14} in Intact Animals

Female rats of the Long-Evans strain, weighing 200 to 220 gm., were used throughout. 1 mg. of emulsified, labeled cholestenone was injected into the femoral vein of each rat. 2 or 3 days later, during which time they were allowed access to food and water, the rats were sacrificed and the distribution of C^{14} in the organs and feces was determined. In the case of liver, intestine, and feces, the C^{14} was further separated into neutral and acidic fractions. The results (Table I) indicate that injected choles-

tenone is rapidly eliminated from the body via feces, primarily in the form of acidic products.

Neutral Sterols—In order to identify the neutral sterols formed from Δ^4-cholestenone, the neutral fractions of liver and feces were chromatographed on alumina columns by elution with increasing concentrations of ethyl ether in hexane, as described above. A typical result is shown in Table II. Identification of the various C^{14} compounds eluted from the column was carried out as follows.

TABLE II

Neutral C^{14} Sterols Found in Liver, Bile, and Feces after Intravenous Injection of Δ^4-Cholestenone-4-C^{14} into Intact and Bile-Cannulated Rats

The neutral sterols extracted from liver, bile, and feces were chromatographed on alumina, as described in the experimental section. The values are given in per cent.

Eluent*	C^{14} peak, compound	Neutral C^{14} sterol recovered at peak					Identity of C^{14} compounds
		72 hrs. after injection into intact rats		48 hrs. after injection into bile-cannulated rats			
		Liver	Feces	Liver	Bile	Feces	
5% ethyl ether in hexane...	I	2.6	1.6	1.8		2.9	Probably cholestanone
10% " " " " ...	II	10	4.0	14.4	2.7		Δ^4-Cholestenone
25% " " " " ...	III	79	91	76.0	92.3	93.9	Cholestanol
Methanol-ethyl ether-hexane (10:60:30).....	IV	8.4	3.6	7.8	5.0	3.2	Unidentified

* The solvents which failed to elute C^{14} from the column are not recorded.

Compound I—The quantity of this substance obtained was too small for identification. However, experiments to be described later indicate that it probably consists in part of cholestanone.

Compound II—The chromatographic behavior of this substance corresponded to that of cholestenone. Its identity was established by adding carrier cholestenone, recrystallizing the mixture, and converting the product to the 2,4-dinitrophenylhydrazone, m.p. 232–233° (22). The identical specific activities of the initial and final products indicate that Compound II is Δ^4-cholestenone.

Compound III—Both cholestanol and cholesterol were eluted at this point. The C^{14} in this fraction was completely precipitated with digitonin. Carrier cholesterol was added to an aliquot of this fraction, and the mixture was brominated as described by Schwenk and Werthessen (23). The

dibromide, after several recrystallizations, was found to be virtually free of C^{14}, which indicates that Δ^4-cholestenone-4-C^{14} is *not* converted to cholesterol to an appreciable extent.

The C^{14} sterol in this fraction was identified as cholestanol as follows: The supernatant liquid from the dibromide preparation was diluted with ethyl ether, washed with sodium bisulfite, and then with water to remove excess bromine. Carrier cholestanol was added to the ethyl ether solution, and the specific activity of the material in solution was determined. Part of the mixture was oxidized to cholestanone, m.p. 130–131° (15), while the rest was acetylated to give the acetate, m.p. 110–111°. The specific activities of these two derivatives were practically identical with that of the starting material. No evidence for any major C^{14} compound other than cholestanol in this fraction was found, either in liver or feces.

Compound IV—The identity of this compound remains unknown.

Acidic Fraction—To obtain information on the nature of the acidic metabolites of Δ^4-cholestenone-4-C^{14} found in feces, aliquots of the fecal extracts were subjected to chromatography by the method of Bergström and Sjövall (21). A number of C^{14} peaks appeared, strongly resembling the results obtained by Bergström and Norman (24) in their studies on acidic fecal metabolites of cholesterol-4-C^{14}. This suggested that the acidic fecal products of the metabolism of Δ^4-cholestenone may be bile acids, perhaps partly degraded by the action of intestinal microorganisms. For this reason, the metabolism of cholestenone in bile-cannulated rats was investigated.

Fate of Δ^4-Cholestenone-4-C^{14} in Bile-Cannulated Rats

Rats provided with bile cannulas (25) were injected intravenously with 1 mg. of cholestenone-4-C^{14}, as described above. Bile was collected, under toluene, at various intervals for 48 hours. The animals were then sacrificed, and the bile, feces plus intestinal content, and liver were analyzed.

In 48 hours, as much as 45 per cent of the injected C^{14} was eliminated in bile (Fig. 1). Solvent fractionation of the hydrolyzed bile showed that 10 to 20 per cent of the biliary C^{14} was present in the neutral fraction, and the rest in the acidic fraction (bile acids). In this period, only 2 to 6 per cent of the C^{14} was recovered in the feces, virtually all of which was in the neutral fraction. 6 to 10 per cent remained in the liver, largely in the neutral fraction. Thus, bile acids are the major products of the metabolism of Δ^4-cholestenone in the rat.

Neutral Sterols—The neutral sterols of liver, bile, and feces were further fractionated on alumina columns and identified as described in the preceding section (Table II).

Bile Acids—In an attempt to identify some of the bile acids formed from

Δ^4-cholestenone-4-C^{14}, a large pooled bile sample was fractionated by the method of Bergström and Sjövall (21). Two C^{14} peaks were obtained, the first corresponding in position to the *trihydroxy* bile acids and the second to the *dihydroxy* bile acids. This suggested that cholic acid and chenodeoxycholic acid might be products of cholestenone metabolism, but the supposition proved incorrect on further study.

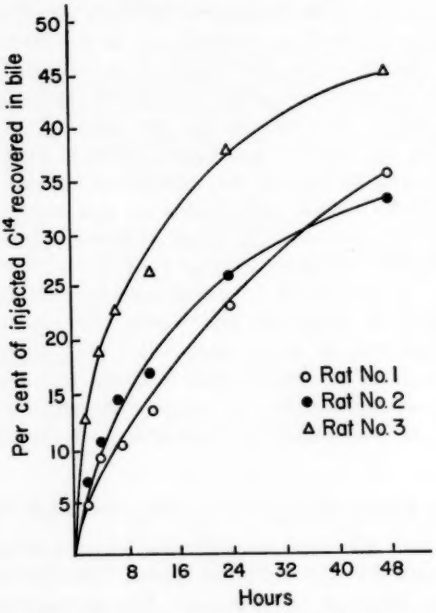

FIG. 1. Cumulative excretion of C^{14} in bile after injection of Δ^4-cholestenone-4-C^{14} into rats provided with bile cannulas.

Carrier cholic acid was added to an aliquot of the *first* peak, and the mixture was recrystallized several times. The virtual absence of C^{14} from the final product indicates that the C^{14} compound under consideration is not cholic acid. By a similar technique it was found that the C^{14} substance at the *second* peak is neither deoxycholic acid nor chenodeoxycholic acid, though in the latter case conversion to the diacetate (16) was necessary in order to remove all of the C^{14}. Thus, it must be concluded that, although Δ^4-cholestenone is degraded to bile acids, these are not identical with the normal bile acids of the rat.

Fig. 2 represents a time study of the incorporation of C^{14} into bile acids.

Whole bile samples collected at various times were analyzed by filter paper chromatography, and radioautograms were prepared as described previously (20). For purposes of comparison, a typical chromatogram of rat bile sprayed with $SbCl_3$ appears in Fig. 2, *A*. The identification of the color bands has been described in a previous paper (20). In the radioautograms of the first bile sample (Fig. 2, *B;* 0 to 2 hours), five C^{14} bands are visible. The first, located at the front, corresponds to neutral biliary C^{14} sterols, the identification of which was discussed above (Table II).

Compound	R_F	Typical rat bile chromatogram sprayed with $SbCl_3$.	Radioautographs of rat bile samples obtained after the injection of Δ^4-Cholestenone-4-C^{14}		
			Hours after injection		
			0-2	7-12	24-48
Cholesterol etc.	Front	—	—	—	—
Taurochenodeoxycholic acid	0.82	—	—	—	—
Taurocholic acid	0.73	—	—*	—*	—*
compound y	0.59	—	—	—	—
	0.44		—	—	—
	Origin				
		A	B	C	D

FIG. 2. Chromatogram and radioautograms of rat bile samples collected at various times after intravenous injection of Δ^4-cholestenone-4-C^{14}. The C^{14} band, indicated by an asterisk, overlaps, but does not coincide with the color band of taurocholic acid. For purposes of representation this displacement has been exaggerated.

The second, at R_F 0.82, corresponds to the position of taurochenodeoxycholic acid, but, as noted above, it is not identical with that substance. The third, at R_F 0.70, overlaps but does not coincide with the color band of cholic acid. The fourth band, at R_F 0.59, corresponds in position to an unidentified bile acid, Compound Y, previously observed as a metabolite of cholesterol-4-C^{14} (20), but it should not be inferred that these two C^{14} substances are identical. The fifth band, at R_F 0.44, does not correspond to any previously noted band.

As the excretory process is followed for longer periods, C^{14} gradually fades from most of the bands (Fig. 2, *C;* 7 to 12 hours) until finally the band at R_F 0.70 becomes the dominant C^{14} bile acid produced from cholestenone (Fig. 2, *D;* 24 to 48 hours).

It was observed that the C^{14} of injected cholesterol-4-C^{14} was incorporated into dihydroxy bile acids before it appeared in the trihydroxy bile acids, and that the C^{14} faded first from the dihydroxy acids (20). No such sequence of appearance or disappearance of the C^{14} of cholestenone-C^{14} was observed in the present study.

Conversion of Δ^4-Cholestenone to Cholestanol by Liver Homogenates

Δ^4-Cholestenone-4-C^{14} was incubated with fortified liver homogenates, and the neutral sterols were isolated by solvent extraction and fractionated

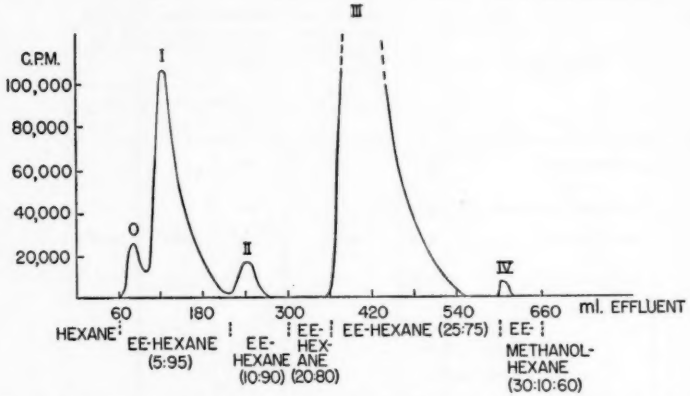

FIG. 3. Chromatographic separation of the products of Δ^4-cholestenone-4-C^{14} metabolism in liver homogenates. 1 mg. of Δ^4-cholestenone-4-C^{14} was incubated at 37° with 10 ml. of rat liver homogenate, 1 ml. of 8×10^{-3} M ATP, and 1 ml. of 1.5×10^{-2} M DPN. The mixture was shaken continuously for 1 hour in an atmosphere of 95 per cent O_2-5 per cent CO_2. After incubation, the mixture was hydrolyzed with alkali and the neutral sterols were isolated by solvent extraction and analyzed by chromatography on alumina columns. The fraction volume was 6 ml. The roman numerals refer to the designation of the C^{14} compounds eluted. EE, ethyl ether.

on alumina columns. Typical results are shown in Fig. 3 and Table III. Δ^4-Cholestenone-4-C^{14} is rapidly metabolized by liver homogenates, cholestanol being the main product. Generally, in 1 hour, about 85 per cent of the added labeled cholestenone was converted to other compounds.

Identification of the C^{14} compounds is discussed below.

Compound O—The possibility that this compound is coprostanone was considered. However, when carrier coprostanone was added, and the mixture converted to the tetrahydrocarbazole, m.p. 194–195°, the derivative was not radioactive. The nature of Compound O is not known.

Compound I—This C^{14} peak was identified as cholestanone. Carrier cholestanone was added to a sample of the eluate. Part of the mixture

was then converted to the tetrahydrocarbazole, m.p. 180–181° (26), and the rest to 2-bromocholestanone, m.p. 164–165° (27). The specific activities of both derivatives were about 80 per cent of the initial specific activity, and remained unchanged upon further recrystallization. Thus, about 80 per cent of the C^{14} in this peak consists of cholestanone.

Compound II—Compound II was identified as unmetabolized Δ^4-cholestenone by means of the 2,4-dinitrophenylhydrazone as described above.

Compound III—This compound was shown to be largely cholestanol, as described above. Again, no C^{14} was found in cholesterol.

TABLE III

Neutral C^{14} Sterols Found after Incubation of Δ^4-Cholestenone-4-C^{14} and Cholestanone-4-C^{14} with Fortified Liver Homogenates

0.1 mg. of C^{14} sterol incubated with 2 ml. of homogenate, 0.2 ml. of ATP, and 0.2 ml. of DPN for 1 hour at 37° in an atmosphere of 95 per cent O_2-5 per cent CO_2. After the incubation, the sterols were isolated by solvent extraction and fractionated on alumina columns.

C^{14} sterol incubated	Experiment No.	Per cent neutral C^{14} sterol recovered* as				
		Compound O	Cholestanone	Δ^4-Cholestenone	Cholestanol	Compound IV
Δ^4-Cholestenone-4-C^{14}	1	7.2	10.8	16.0	62.4	3.6
	2	5.5	9.0	17.1	64.9	3.5
	3	2.2	16.4	1.9	78	1.5
Cholestanone-4-C^{14}	1		34		45	21
	2		52		40	8
	3		42		52	6

* Almost all of the added C^{14} was recovered in the neutral C^{14} sterol fraction.

Compound IV—At present unidentified, this compound no doubt corresponds to the "Compound IV" noted in the experiment with intact rats (Tables II and III).

We were unable to demonstrate the formation of C^{14} bile acids under the conditions of the present study.

Metabolism of Cholestanone-4-C^{14} by Liver Homogenates

The formation of cholestanone-C^{14} in liver homogenates incubated with Δ^4-cholestenone-4-C^{14} is in accord with the suggestion (3) that this compound is an intermediate in the reduction of Δ^4-cholestenone to cholestanol. For this reason, cholestanone-4-C^{14} was incubated with liver homogenates exactly as described above for cholestenone. The cholestanone-4-C^{14}

was rapidly metabolized (Table III). In 1 hour, about 50 per cent of the added C^{14} was converted to cholestanol, identified as described above, and to traces of "Compound IV."[2] The remainder was unmetabolized cholestanone. A typical analytical result is shown in Fig. 4.

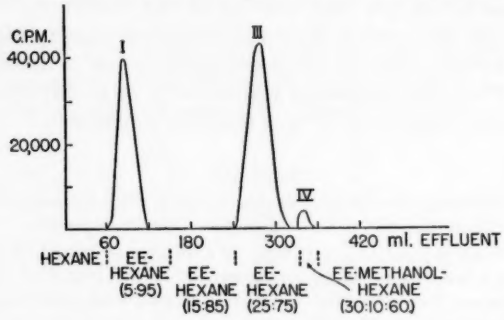

FIG. 4. Chromatographic separation of the products of cholestanone-4-C^{14} metabolism in liver homogenates. 0.1 mg. of cholestanone-4-C^{14} was incubated at 37° with 2 ml. of liver homogenate, 0.2 ml. of 8×10^{-3} M ATP, and 0.2 ml. of 1.5×10^{-2} M DPN. The mixture was shaken continuously for 1 hour in an atmosphere of 95 per cent O_2-5 per cent CO_2. After the incubation, the mixture was hydrolyzed with alkali, and the neutral sterols were isolated by solvent extraction and analyzed by chromatography on alumina columns. The fraction volume was 10 ml. The roman numerals refer to the designation of the C^{14} compounds eluted. EE, ethyl ether.

DISCUSSION

The major portion of the C^{14} of intravenously injected Δ^4-cholestenone-4-C^{14} is rapidly eliminated by intact rats via feces. The fecal C^{14} consists of two fractions: an acidic fraction that contains about 60 to 70 per cent of the fecal C^{14}, and a neutral one.

When Δ^4-cholestenone-4-C^{14} is injected into rats provided with bile cannulas, all the acidic metabolites are recovered in bile. This observation, together with the chromatographic behavior of these acidic compounds, indicates that they are bile acids.[3] At least four C^{14} bile acids were observed in the bile of these rats, but none was identical with either of the major bile acids of the rat, taurocholic acid and taurochenodeoxycholic acid. Thus, the bile acid end-products of Δ^4-cholestenone catabolism are

[2] Although the chromatographic behavior of "Compound IV" was identical with that of Compound IV (Tables II and III), they need not be the same compound.

[3] Bile acids are defined here as acidic steroids eliminated in bile, regardless of structure and length of side chain. It is reasonable to believe, however, that we are dealing with C-24 compounds.

not identical with those of cholesterol metabolism (20, 28), and hence the suggestion of Rosenheim and Webster (1) is untenable. None the less, the chromatographic behavior of these unidentified bile acids warrants the suggestion that they, too, are conjugated with taurine and possess structures similar to those of cholic acid, chenodeoxycholic acid, and Compound Y. Since all the neutral 27 carbon sterols studied to date (cholesterol (20, 28), epicholesterol (18), cholestanol),[4] as well as ergosterol (29), undergo degradation to bile acids, it would appear that such degradation is a prominent feature of the catabolism of sterols possessing degradable side chains.

The neutral C^{14} sterols found in feces after the injection of Δ^4-cholestenone-4-C^{14} into intact rats have in part been identified. The major component is cholestanol, accompanied by small amounts of unmetabolized cholestenone and probably cholestanone, and at least one unidentified sterol. The same C^{14} sterols were also observed in the livers of these rats. In agreement with the work of Stokes *et al.* (10), only negligible quantities of C^{14} were found in cholesterol.

It should be noted that, under our experimental conditions, cholestenone is not converted to coprostanol to a significant extent. This, however, is not surprising since the latter transformation probably takes place in the intestinal lumen (2), whereas in the present experiments little of the injected cholestenone-C^{14} reached the intestine as such. The main site of metabolism of injected cholestenone is clearly the liver.

In a comparison of the C^{14} sterol composition of the bile and feces after injection of Δ^4-cholestenone-4-C^{14} into rats provided with bile cannulas, it is found that cholestenone and cholestanol reach the intestine via the bile. In addition it is shown, in agreement with earlier findings of Schoenheimer and von Behring (30), that cholestanol is secreted directly across the gut wall.

In the hope of learning more about the pathway of conversion of cholestenone to cholestanol, this reaction was studied in liver homogenates. The reaction proceeds readily, and considerable quantities of cholestanone and an unidentified substance, Compound O, accumulate. The observation that cholestanone-4-C^{14} is also readily converted to cholestanol in this system lends support to the suggestion of Bloch (3) that the former is an intermediate in the reduction of Δ^4-cholestenone to cholestanol. Numerous attempts to establish this point by the isotope dilution technique failed in our hands, owing to difficulties encountered in emulsifying relatively large amounts of sterols.

[4] Harold, F. M., Chapman, D. D., and Chaikoff, I. L., unpublished observations.

SUMMARY

1. Δ^4-Cholestenone-4-C^{14} was injected intravenously into rats. In 67 hours, 50 per cent of the C^{14} was eliminated in feces. Of the fecal C^{14}, about 65 per cent was recovered in an acidic fraction apparently composed of bile acids. The rest was in the neutral sterol fraction. Cholestanol was the predominant neutral sterol formed from cholestenone, both in liver and in feces. In addition, cholestenone and two minor, unidentified products were noted in both liver and feces. The incorporation of C^{14} into hepatic and fecal cholesterol was negligible.

2. When labeled cholestenone was injected into rats provided with bile cannulas, as much as 45 per cent of the injected C^{14} was recovered in the bile in 48 hours. Most of the biliary C^{14} was in the bile acid fraction. At least four C^{14} bile acids were detected, but none of these is identical with the major bile acids of the rat (taurochenodeoxycholic and taurocholic acids). The biliary neutral sterols consisted of cholestenone and cholestanol, whereas the fecal C^{14} was mostly in cholestanol.

3. When Δ^4-cholestenone-4-C^{14} was incubated with liver homogenates, rapid conversion to cholestanol was observed. In addition, cholestanone and two unidentified products were observed.

4. Cholestanone-4-C^{14} was readily reduced to cholestanol by liver homogenates.

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THE EXTRAHEPATIC METABOLISM OF PROGESTERONE IN RATS*

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Investigators have failed to find high concentrations of progesterone in tissues of animals secreting relatively large quantities of progestational substance (1–3); and metabolic studies in which progesterone-21-C^{14} was used (4, 5) have shown that neither the hormone nor its metabolites were stored in appreciable quantities in the tissues of rats. It has been concluded (4), therefore, that the metabolism of progesterone after its synthesis in endocrine glands is continuous and rapid. There is much experimental evidence to suggest that the liver is the major site for the biological inactivation of progesterone (1). However, except for certain studies *in vitro* with isolated tissues and organs, no investigation of the extent of extrahepatic metabolism has been made. Therefore it seemed of interest to investigate the fate of circulating progesterone in animals deprived of liver, spleen, and gastrointestinal tract to determine whether these are the sole sites of its metabolism. In addition, it was considered possible that without the excretory action of the liver there might be a greater tendency for localization or deposition of the hormone in certain tissues. Finally, it seemed that, if extrahepatic metabolism did occur, the products might have physiological importance bearing on the mechanism of hormone action of progesterone or on its rôle as a precursor of other hormonally active substances.

EXPERIMENTAL

Young adult rats (200 to 250 gm.) of the Sprague-Dawley strain were used in all experiments. Liver, spleen, and gastrointestinal tract were removed from all the experimental animals according to the procedure described by Ingle (6). Penicillin (100,000 units) and streptomycin (20 mg.), dissolved in 0.9 per cent saline, were injected intraperitoneally at the conclusion of the operation to prevent septicemia. Animals allowed to survive longer than 4 hours were given intravenous administrations of glucose plus antibiotics at 4 hour intervals.

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XUM

Progesterone-4-C^{14} was administered intravenously in doses of 780,000 to 800,000 c.p.m. (49 to 50 γ) as a solution in either 25 per cent bovine serum albumin or in propylene glycol. The purity of the progesterone was checked both by its homogeneity on chromatography and by its constant specific activity on repeated crystallization.

The animals were kept in metabolism cages, excreta were collected, and, after the desired time intervals, the animals were anesthetized with ether and sacrificed by withdrawal of blood from the abdominal aorta. Aliquots of the drawn blood plated on nutrient agar showed no bacterial growth in all cases.

Tissues were removed and homogenized with sufficient distilled water to

TABLE I

Reliability of Sampling and Counting Tissues and Urine

Material plated	Volume plated	Sample thickness	Measured radioactivity (R_T) $\times 10^{-4}$	Thickness correction (R_T/R_0*)
	ml.	*mg. per sq. cm.*	*c.p.m.*	
Kidney homogenate	0.05	0.35 ± 0.02†	1.84 ± 0.04†	0.842
" "	0.10	0.62 ± 0.03	1.65 ± 0.05	0.757
" "	0.40	2.24 ± 0.09	1.16 ± 0.02	0.533
Urine	0.05	0.60 ± 0.01	1.52 ± 0.04	0.704
"	0.10	1.19 ± 0.003	1.36 ± 0.02	0.625
"	0.40	4.53 ± 0.005	0.78 ± 0.02	0.356

* R_0 = amount of radioactivity present in each sample = $2.18 \pm 0.03 \times 10^4$ c.p.m.

† Each value represents the mean and the standard error of the mean for six determinations.

produce a 10 per cent homogenate. Aliquots (0.05 to 0.4 ml.) of tissue homogenates, blood, urine, and intestinal contents from intact control rats were plated directly on aluminum plates for determination of isotope content. All the samples were counted in internal flow counters long enough to give a standard error of 10 per cent or less. The reliability of sampling and counting such samples is indicated by the data contained in Table I. Correction factors for loss of radioactivity due to internal absorption of the sample were obtained from a plot of the thickness correction *versus* the sample thickness.

Excretion of Radioactivity—After progesterone-4-C^{14} administration to intact male rats, radioactivity was rapidly eliminated from the tissues in agreement with the findings of others (4). The rates of excretion of radioactivity from intact and eviscerated rats are compared (Table II) and show that, within 1 hour, radioactivity appeared in the urine and duodenal contents of the control, while none was present in the urine of the eviscerated

rats. The decrease in radioactivity of the duodenal contents of the control animals from 5 to 22 hours indicates that the major portion of biliary excretion was completed prior to 22 hours.

The *distribution of radioactivity* among tissues of intact and eviscerated male rats was also determined at 1, 5, and 22 hour intervals after the injection of progesterone-4-C^{14}. Radioactivity was detectable in several tissues of the intact animals up to 5 hours, with the highest concentration being present in the liver (Table III). However, after 22 hours no isotope was measurable in any tissue. By contrast, the concentration of C^{14} was considerably higher in tissues of the eviscerated rats and remained high during the 22 hours of the experiment. A tendency for radioactivity to accumulate in kidneys and adrenals was indicated.

TABLE II

Excretion of Radioactivity after Intravenous Injection of Progesterone-4-C^{14} to Male Rats*

Rat preparation and excretion route		After injection					
		1 hr.		5 hrs.		22 hrs.	
		c.p.m.	*per cent*†	*c.p.m.*	*per cent*†	*c.p.m.*	*per cent*†
Intact rats	Urine	27,000	3.4	31,600	4.0	46,800	5.9
" "	Duodenal contents	13,520	1.7	226,000	28.3	23,800	3.0
Eviscerated rats	Urine	0	0	3,900	0.5	5,700	0.7

Radioactivity in the urine is that accumulated over 1, 5, and 22 hours after injection. "Duodenal contents" refers to that of the upper third of the small intestine, at the time of sacrifice.

* 800,000 c.p.m. (50 γ).

† Percentages of administered C^{14}.

The rôle of the adrenals and kidneys in the distribution of radioactivity in the eviscerated female was next investigated. For this experiment female rats were separated into four groups of five animals each and subjected to surgery according to the following plan: Group A, eviscerated only; Group B, eviscerated and nephrectomized; Group C, eviscerated and adrenalectomized; and Group D, eviscerated, nephrectomized, and adrenalectomized. Tissues were removed from the rats 1 hour after injection of progesterone-4-C^{14}, and the isotope content of each tissue was determined. The results are presented in Table IV. No accumulation of radioactivity was evident in either uterus or ovaries, since in most groups the isotope content of these tissues was equal to or less than the value for blood. The tendency for accumulation in kidneys and adrenals observed in the study with males was substantiated in this experiment. However, the removal

TABLE III

*Change with Time of Isotope Content of Tissues after Intravenous Injection of Progesterone-4-C¹⁴ to Intact and Eviscerated Male Rats**

The values are given in counts per minute per mg.

Tissue	After injection											
	1 hr.				5 hrs.				22 hrs.			
	Intact		Eviscerated		Intact		Eviscerated		Intact		Eviscerated	
		per cent†		per cent†		per cent†		per cent†		per cent		per cent†
Blood	4.2	0.53	47.0	3.0	1.5	0.25	14.8	1.5	0	0	31.6	2.5
Liver	13.9	2.6			11.4	2.7			0	0		
Kidneys	9.1	0.47	38.0	2.6	4.5	0.18	29.3	1.48	0	0	28.4	1.79
Adrenals	4.8	0.04	28.0	0.05	4.9	0.04	30.0	0.08	0	0	77.5	0.05
Testes	2.2	0.19	21.6	0.83	3.0	0.21	13.7	0.58	0	0	20.4	0.97
Seminal vesicles			2.7	0.13	1.1	0.04	1.1	0.05	0	0	6.5	0.07
Submaxillary gland	0	0	20.5	0.21			20.8	0.18			8.8	0.21
Thymus	0	0	25.4	0.30			13.3	0.14			20.0	0.14

* 800,000 c.p.m. (50 γ). Each column presents the data from a single animal.
† Percentages of the administered C¹⁴ found in each tissue.

TABLE IV

*Average Distribution of Radioactivity in Tissues of Female Rats 1 Hour after Intravenous Administration of Progesterone-4-C¹⁴**

Tissue	Eviscerated only		Eviscerated; nephrectomized		Eviscerated; adrenalectomized		Eviscerated; adrenalectomized; nephrectomized	
	c.p.m. per mg.	per cent†	c.p.m. per mg.	per cent†	c.p.m. per mg.	per cent†	c.p.m. per mg.	per cent†
Blood	24.9 ± 1.8	1.6	25.9 ± 5.1	1.7	21.7 ± 2.3	1.4	30.8 ± 3.1	1.9
Kidneys	58.6 ± 4.3	2.4			55.8 ± 6.3	1.8		
Adrenals	90.2 ± 13.1	0.07	61.8 ± 17.2	0.05				
Uterus	22.0 ± 3.5	0.13	18.8 ± 4.0	0.14	14.4 ± 5.6	0.15	16.6 ± 4.1	0.17
Ovaries	30.9	0.07	23.8	0.07	15.1	0.09	32.4	0.10

* 780,000 c.p.m. (49 γ). Specific radioactivities are expressed per unit of dry weight of tissue. Each group contained five animals; the mean values and the standard error of the mean are given. Ovaries removed from members of a group were pooled prior to assay.
† Percentages of the administered C¹⁴ found in each tissue.

of the kidneys or adrenals did not significantly affect the distribution of isotope among other tissues.

In the studies of eviscerated male and female rats, the radioactivity in organs and tissues other than those listed in Tables III and IV was also determined. These included pituitary gland, brain, heart, lung, lymph nodes, salivary glands, prostate, muscle, and peritoneal fat. None of these tissues had a specific radioactivity greater than the value for blood in the corresponding animal.

Separation of Steroids—The chemical nature of the material containing the labeled carbon atom in the tissues of the female rats comprising Groups A, B, C, and D mentioned previously was investigated. Tissues to be studied, having been removed, homogenized, and assayed for radioactivity, were extracted by a modification of the Zaffaroni dialysis-extraction technique (7). In this procedure the tissue suspension was brought to 50 per cent methanol concentration and dialyzed through a cellophane membrane against 50 per cent methanol plus a layer of ethylene dichloride. Extraction was continued for 96 hours with three changes of ethylene dichloride. By this technique it was possible in one operation to obtain steroid fractions which were sufficiently free of pigmented material and fat to be chromatographed directly on paper. In Table V are recorded recoveries of radioactivity from crude tissue homogenates obtained by the dialysis-extraction technique.

TABLE V

Recoveries of Radioactivity Obtainable with Zaffaroni Dialysis-Extraction Technique from Tissue Homogenates of Female Eviscerated Rats comprising Groups A, B, C, and D

Tissue	No. of homogenates extracted	Recovery*
		per cent
Kidney	10	46 ± 9
Blood	20	51 ± 3
Uterus	20	54 ± 5
Ovaries	20	65 ± 8

* Mean recoveries and the standard errors of the mean.

The ethylene dichloride extracts were evaporated to dryness under nitrogen, redissolved in a small volume of methanol-ethylene dichloride, and applied to narrow strips of Whatman No. 1 filter paper for chromatography by the Zaffaroni (8) method. Spots of radioactivity were detected by counting consecutive 2 cm. pieces of the chromatograms directly in flow counters. It may be seen from Fig. 1 that the progesterone-4-C^{14} employed in these studies was chromatographed as a single spot. Also shown in Fig.

1 are the results of two control experiments in which progesterone-4-C^{14} was homogenized with tissue from uninjected rats. According to these results, no *in vitro* metabolism of progesterone occurred during either homogenization or extraction of the tissues.

In contrast to the chromatograms in Fig. 1, chromatography of extracts of all the tissue homogenates and blood samples from the female eviscerated rats gave results similar to those illustrated for the ovaries from these animals (Fig. 2). Regardless of the tissue from which the extract was ob-

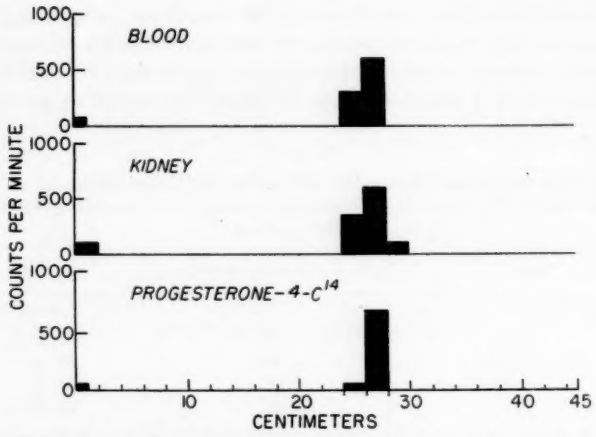


FIG. 1. The two top sections of the figure give chromatographic results of control experiments in which progesterone-4-C^{14} was homogenized with blood and kidneys from uninjected rats. Progesterone was extracted from tissues by dialysis-extraction technique. In the bottom part of the figure is the chromatogram of a solution of progesterone-4-C^{14} in propylene glycol. All three chromatograms were developed for 7 hours in heptane-formamide (8).

tained, the chromatograms usually contained five zones of radioactivity. However, if either the amount of fatty contamination or the quantity of steroid mixture applied to the paper was large, there was a tendency for Zones 3 and 4 not to be resolved.

Because of incomplete separation of the zones in some cases, only rough estimations could be made of the amounts of radioactivity in each of the five zones separated by chromatography (Table VI). However, these estimations do indicate differences in the chromatographic distribution patterns of the various tissues. For example, the kidney extracts contained a larger proportion of non-polar material, Zone 5, than did the other tissues, ovaries were rich in the material constituting Zone 4, and the highest

concentration of radioactivity obtained from any of the tissues appeared in Zone 1 of the adrenal extracts.

Each zone of radioactivity was eluted from the chromatograms with ab-

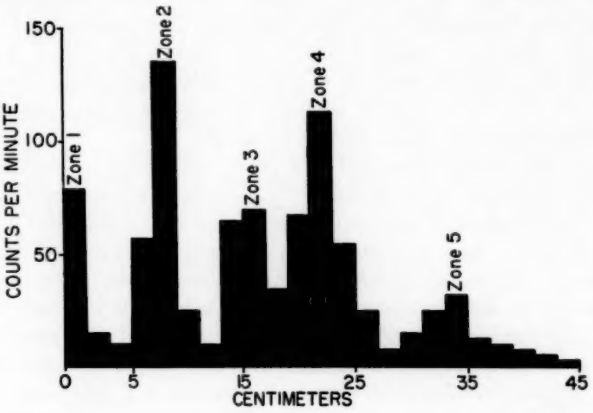


FIG. 2. Chromatogram of extracts from rat ovaries obtained from eviscerated rat Group D. Hexane-formamide (8) system was used with a running time of 5 hours.

TABLE VI

Average Distribution of Radioactive Constituents in Extracts of Tissues of Female Eviscerated Rats As Determined by Chromatography

Tissue	Per cent total radioactivity on chromatogram				
	Zone 1	Zone 2	Zone 3	Zone 4	Zone 5
Blood	23	27	20	21	9
Kidneys	27	6	18	16	33
Uterus	25	13	20	20	22
Ovaries	12	27	17	33	11
Adrenals	49	8	11	24	8

Tissue extracts were chromatographed in the Zaffaroni (8) hexane-formamide for 5 hours. Radioactivity was measured on 2 cm. sections of the chromatogram in a flow counter.

solute methanol and chromatographed again in an appropriate system with either the Zaffaroni (8) or the Bush (9) technique. Known steroids were chromatographed alongside as an aid in characterization, and a comparison of the mobilities of the radioactive substances with the mobilities of known steroids permitted an estimation of the number of oxygen atoms present in the unknown. Zone 1 was chromatographed in benzene-55 per

cent aqueous methanol and separated into three components with R_F values of 0.18, 0.32, and 0.80. When chromatographed separately in benzene-55 per cent aqueous methanol or chloroform-formamide, these three components could not be further divided. They were considered to be homogeneous compounds and were designated Compounds 1a, 1b, and 1c. All three compounds absorbed ultraviolet light in the region of 240 mμ, indicating the probable presence of the Δ^4-3-ketone grouping. Compounds 1a and 1b moved in similar manner to steroids containing 5 oxygen atoms; Compound 1c moved like an O_4 steroid.

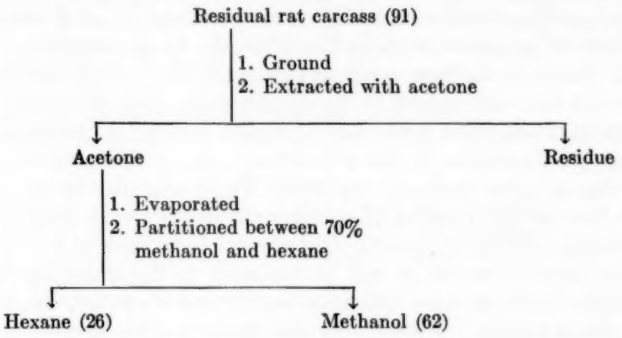
Zone 2 was chromatographed as a single spot, both in heptane-formamide (R_F, 0.22) and in Skellysolve C-85 per cent aqueous methanol (R_F, 0.27). This compound, designated Compound 2, also absorbed ultraviolet light. It gave a dark blue color with iodine (10) and formed an acetate. The acetate had an R_F of 0.84 in heptane-formamide. The mobility of Compound 2 in the two systems used indicated that it had only 2 oxygen atoms. By chromatography it could not be identified with any of the following known compounds: Δ^4-pregnen-20β-ol-3-one, Δ^4-pregnene-3,11,20-trione, Δ^4-pregnen-17-ol-3,20-dione, Δ^4-pregnene-3,6,20-trione, Δ^4-pregnen-6β-ol-3,20-dione, pregnane-3α,20α-diol, $\Delta^{4,6}$-pregnadiene-3,20-dione, $\Delta^{4,11}$-pregnadiene-3,20-dione, Δ^4-pregnen-20β-ol-3,11-dione, and allopregnan-3α-ol-20-one.

Zones 3 and 4 could be completely separated by chromatography in heptane-formamide if the solvent were allowed to run twice the time necessary to reach the end of the paper. Neither of these zones could be further divided by chromatographing in heptane-formamide (R_F, 0.39 and 0.45), Skellysolve C-85 per cent aqueous methanol (R_F, 0.50 and 0.59), or Skellysolve C-95 per cent aqueous methanol (R_F, 0.34 and 0.45). Consequently, the two substances were referred to as Compounds 3 and 4. Compound 3 did not absorb ultraviolet light. It formed an acetate which had an R_F of 0.9 in heptane-formamide. Compound 4 did absorb ultraviolet light, and it could not be acetylated.

Zone 5 was chromatographed as a single spot in heptane-formamide (R_F, 0.63) and in Skellysolve C-95 per cent aqueous methanol (R_F, 0.49). It was therefore considered homogeneous and was called Compound 5. This compound did not absorb ultraviolet light and did not form an acetate.

The chromatographic mobilities of the seven substances just described are incompatible with those of all known conjugated steroids; such compounds would not move from the origin in any of the systems used here. Since only about 4 per cent of the administered isotope was present in the blood and other specific organs analyzed, it seemed important to determine the nature of the radioactive compounds in the remainder of the carcass. Consequently, the residual carcasses were ground thoroughly in a meat

grinder and extracted with several changes of boiling acetone until no radioactivity was detectable in the tissue. This acetone extract was prepared for chromatography by the accompanying purification Scheme 1. It may be seen that 91 per cent of the administered radioactivity was recovered from the residual carcass. Partitioning the carcass extract between 70 per cent methanol and hexane yielded a methanol fraction which could be successfully chromatographed, although it contained only 62 per cent of the radioactivity originally injected. Only the seven compounds described earlier were found in this material. The hexane fraction, which contained 26 per cent of the radioactivity injected, was not as thoroughly characterized because of the large amount of fat which it contained; however, chromatography of aliquots of the hexane fraction showed only the presence of relatively non-polar compounds. The results indicate that no significant amount of polar, water-soluble conjugates, such as are ordinarily found in the excreta of intact animals, is formed in the absence of the viscera.



SCHEME 1. Flow diagram of extraction procedure used to separate constituents from eviscerated rat carcasses. The figures in parentheses represent the per cent of the administered dose.

DISCUSSION

The distribution of radioactivity in normal rats is compatible with the view that metabolites of progesterone accumulate rapidly in the liver from which they are discharged mainly via the bile into the intestinal tract and, to a lesser extent, into the blood eventually to be excreted in the urine (4, 5). It would seem that the excretion of metabolites depends primarily on the formation in the liver of highly water-soluble steroid conjugates (1). The present investigation indicates that no detectable conjugation with

such compounds as glucuronic and sulfuric acids takes place in the absence of the liver, since 91 per cent of administered radioactivity was accounted for in the eviscerated rats as compounds of low water solubility and relatively low polarity. The present study also shows that associated with the failure to form water-soluble conjugates is a concomitant retention of metabolites in the peripheral tissues of eviscerated rats.

Perhaps of greater importance than understanding the mechanisms of excretion is a consideration of the differences between "metabolism" and "inactivation" (1). Evidence seems well founded that the liver is the major site for the biological inactivation of progesterone (1). However, in view of the extensive extrahepatic metabolism observed in this study and the hormonal activity of peripherally administered progesterone, an important question would seem to be, "Is the hormonal action of peripherally administered progesterone due to the metabolic changes occurring in extrahepatic tissues, or do these reactions inactivate the hormone but at a relatively slow rate with respect to the changes occurring in the liver?"

A result of this work which has a possible bearing on this point is the finding that destruction of the Δ^4-3-ketone configuration occurs only to a minor degree in the absence of the liver. The inactivation of progesterone by the liver and the capacity of this organ to reduce ring A may be related phenomena. Before any positive statements can be made in answer to the question posed, however, it will be necessary to determine whether the metabolites found have any biological activity and where the observed metabolic changes occur. Work on the identification of the seven compounds reported in this paper is being pursued preparatory to biological testing. As to the site of extrahepatic metabolism, for the present it can only be said that either the chemical transformations occur in all peripheral tissues or they occur in tissues other than kidney and adrenal. However, it seems highly probable that these latter two glands do take some part in progesterone metabolism, since there are differences between them and other tissues in the proportionate distribution of radioactivity among the various metabolites. The proportion of the highly oxygenated compounds present in adrenals could be due to enzyme systems present in this tissue capable of converting progesterone to various adrenal hormones (11, 12).

The authors wish to express their appreciation to Dr. Leo T. Samuels, Head of the Department of Biological Chemistry, for his interest and many valuable suggestions.

SUMMARY

Intravenously administered progesterone is extensively metabolized in 1 hour by eviscerated rats. After this interval, seven compounds could be

isolated from the tissues of these animals. Metabolism occurs with or without the presence of the kidneys or adrenals. No polar metabolites similar to the conjugates with glucuronic or sulfuric acid usually present were found in the eviscerated rats. Five of the seven compounds isolated retain the Δ^4-3-ketone configuration. Further chemical characterization of these compounds is given, but they are not specifically identified.

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THE METABOLISM OF PROGESTERONE TO Δ^4-PREGNEN-20α-OL-3-ONE IN EVISCERATED FEMALE RATS*

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A previous report from this laboratory showed that seven radioactive steroids could be separated from the tissues of eviscerated female rats given progesterone-4-C^{14} 1 hour prior to sacrifice (1). One of these compounds, designated as Compound 2, retained the Δ^4-3-ketone configuration; in addition, it formed an acetate under mild conditions and reacted on paper chromatograms with iodine to give a blue color. A blue reaction with iodine has been shown to be limited to only a few steroids of a large number tested (2). This paper presents the identification of Compound 2 as Δ^4-pregnen-20α-ol-3-one.

EXPERIMENTAL

Isolation and Identification of Compound 2—Five young adult Sprague-Dawley rats weighing approximately 200 gm. each were eviscerated by the Ingle technique (3) and, in addition, were adrenalectomized. After surgery, penicillin and streptomycin dissolved in normal saline were injected intraperitoneally. Twice recrystallized progesterone, m.p. 128–129°, was dissolved in propylene glycol, 50 mg. per ml. The solution was supersaturated at room temperature and had to be warmed to 50° prior to use to prevent crystallization of the steroid. 0.1 ml. of the progesterone solution was injected into the left saphenous vein of each rat, and the animals were allowed to survive for 2 hours after injection. They were then sacrificed, frozen immediately, and the skins and tails removed and discarded. The frozen carcasses were ground together in a meat grinder and extracted with a solvent mixture composed of 25 ml. of ethylene dichloride, 25 ml. of 95 per cent ethanol, and 50 ml. of ethyl acetate. The extraction procedure, repeated seven times, consisted of triturating the ground tissue in approximately 1 liter of solvent at 30–40° and pressing the extract through cotton toweling. Separation of steroids from the carcass extract was accomplished as outlined in the accompanying Scheme 1. Through-

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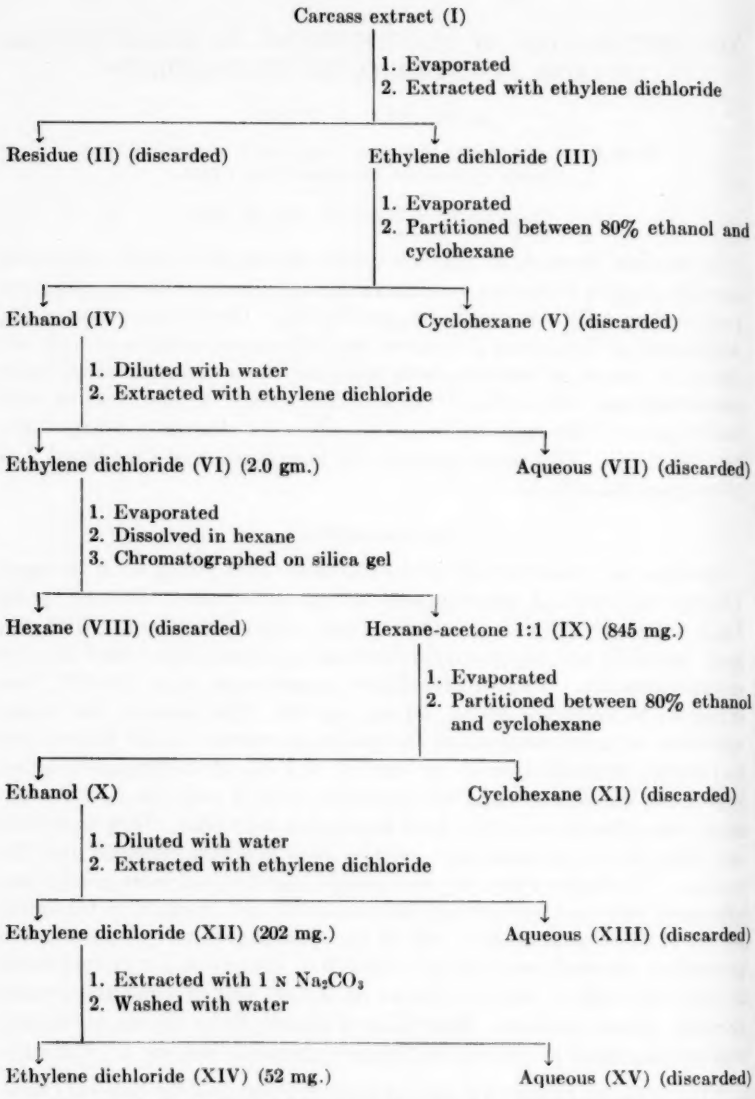
XUM



SCHEME 1. Flow diagram of the procedure used to separate steroids from rat tissues.

out the manipulations all evaporations were carried out *in vacuo* under nitrogen, and the temperature was never allowed to rise above 40°. The ethylene dichloride solution (VI) on evaporation yielded 2 gm. of gummy residue. Yields of steroid-containing fractions obtained from subsequent purification procedures are given in the scheme. Paper chromatographic analysis of fraction XIV indicated about 50 per cent steroid, largely unmetabolized progesterone. A similar study, employing the same extraction and separation procedures but used to separate progesterone-4-C^{14} and its metabolites from rat carcasses, gave a yield, based on radioactivity, of 75 per cent.

Final separation of Compound 2 from other steroids present in fraction XIV was accomplished by chromatography in the Zaffaroni heptane-formamide system (4) by using three filter paper strips, each 17 cm. wide, and allowing 5 hours for development. On examination with ultraviolet light each paper showed three absorption bands: a narrow band at the origin, a sharp discrete band 9 cm. from the origin, and a wider band with its center 25 cm. from the origin. While still wet with formamide, each area was cut out and eluted, the two lower bands with hexane and the material at the origin with ethylene dichloride. Eluates from similar bands in the three chromatograms were then combined.

The mobility of the middle band and its blue reaction with iodine indicated its identity with Compound 2 of our previous studies (1). Work was then directed toward further characterization of this substance. Evaporation of the hexane gave a light yellow crystalline residue. This material was recrystallized once from acetone and yielded 2.57 mg. of colorless material, m.p. 131–158°. The crystalline material was analyzed by infra-red spectroscopy and showed a hydroxyl peak at 2.75 μ and a peak at 6.00 μ indicative of the Δ^4-3-ketone grouping.

Since the R_F of Compound 2 in the hexane-formamide system was roughly comparable to that of pregnan-3α-ol-20-one and yet the compound absorbed ultraviolet light in the 240 mμ region characteristic of Δ^4-3-ketosteroids, it seemed probable that it was produced by the biological reduction of the 20-carbonyl group of progesterone to yield Δ^4-pregnen-20-ol-3-one. Consequently 80 γ of Δ^4-pregnen-20α-ol-3-one[1] and 70 γ of Compound 2 were chromatographed on separate strips of paper 2 cm. wide in formamide-hexane for 10 hours. Spots were located by ultraviolet scanning. A facsimile of the chromatogram is given in Fig. 1, Strip 1. The strips were treated with iodine, and both spots gave the same blue color reaction.

Infra-red spectroscopic analysis was made of the Δ^4-pregnen-20α-ol-3-

[1] Kindly furnished by Dr. A. Butenandt, Max Planck Institute, Tübingen, Germany.

one obtained from Dr. Butenandt and of crystalline Compound 2. It was found that the position and intensity of the absorption bands agreed within the limits of accuracy of the spectrometric records. Table I contains the absorption maxima of both compounds.

The conditions previously used for the oxidation of Δ^4-pregnen-20α-ol-3-

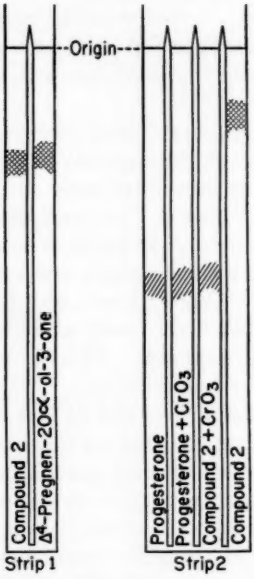


FIG. 1. Strip 1 is a sketch of the paper chromatogram which compares the mobilities of Compound 2 and Δ^4-pregnen-20α-ol-3-one, heptane-formamide system for 10 hours at room temperature. Strip 2 demonstrates the oxidation of Compound 2 to progesterone as shown by chromatography of the product in heptane-formamide for 7 hours. Cross-hatched and diagonally lined areas indicate, respectively, the blue and the yellow colors produced by treatment of the chromatograms with iodine.

one to progesterone (6) were applied to Compound 2 and resulted in its conversion to the hormone. 100 γ each of Compound 2 and of progesterone were dissolved in 0.025 ml. of glacial acetic acid containing 45 γ of CrO_3 and were allowed to remain at room temperature for 30 minutes. At the conclusion of this time the two solutions were chromatographed for 7 hours in heptane-formamide together with untreated Compound 2 and progesterone. The chromatogram is reproduced in Fig. 1, Strip 2. All four spots were clearly visible as dark areas when viewed by ultraviolet light.

The first three spots gave a yellow color with iodine, while the fourth, due to the untreated Compound 2, gave a blue color with this reagent.

The mother liquor obtained from the recrystallization of Compound 2 was evaporated to dryness. 4 drops of pyridine and 6 drops of acetic anhydride were added. The mixture was allowed to stand overnight at room temperature, and was then chromatographed on two strips of filter paper, each 17 cm. wide, in a modified Zaffaroni system. In this system the paper was impregnated with 80 per cent formamide in methanol, blotted, and the chromatogram developed with pentane at 1°. On scanning

TABLE I

Infra-Red Absorption Maxima

Compound 2	Δ^4-Pregnen-20α-ol-3-one	Compound 2 acetate	Δ^4-Pregnen-20α-ol-3-one monoacetate*
μ	μ	μ	μ
2.75, 3.41, 3.48, 6.00, 6.19, 6.92, 7.28, 7.39, 7.53, 8.67, 8.73, 9.05, 9.89, 10.02, 10.57, 11.11, 11.56	2.70, 3.44, 3.51, 6.00, 6.19, 6.92, 7.27, 7.38, 7.52, 8.69, 8.72, 9.01, 9.92, 10.02, 10.56, 11.10, 11.58	5.76, 5.96, 7.33, 7.53, 7.66, 7.91, 8.04, 8.45, 8.64, 8.71, 8.93, 8.97, 9.06, 9.30, 9.47, 9.82, 10.54, 10.75, 12.15, 12.89, 14.66	5.76, 5.96, 7.51, 7.63, 7.88, 8.02, 8.42, 8.62, 8.69, 8.89, 8.96, 9.05, 9.28, 9.44, 9.79, 10.53, 10.72, 12.10, 12.86, 14.63

In infra-red studies a Perkin-Elmer recording spectrophotometer model 21 was employed. Compound 2, 2.5 mg. per 0.4 ml., and Δ^4-pregnen-20α-ol-3-one, 1.0 mg. per 0.1 ml., were analyzed in chloroform. The analysis of Δ^4-pregnen-20α-ol-3-one required the use of a micro cell. Acetate of Compound 2, 3.0 mg. per 0.4 ml., was analyzed in carbon disulfide.

* Data taken from Hirschmann *et al.* (5).

with ultraviolet light, bands 6 to 7 cm. wide were observed with centers at R_F 0.3. These bands were cut from the formamide-impregnated papers and eluted with heptane. The two solutions were combined and evaporated to a crystalline residue. This was recrystallized from pentane, m.p. 105–134°. Infra-red spectroscopic analysis (Table I) gave absorption maxima corresponding with those published for Δ^4-pregnen-20α-ol-3-one acetate, m.p. 141–142° (5).

While sufficient recrystallization to obtain a sharp melting point was impossible because of the limited amount of material, Compound 2 was identified by its mobility and infra-red absorption, by oxidation to progesterone, and by acetylation to a compound giving the infra-red spectrum of Δ^4-pregnen-20α-ol-3-one acetate. Not enough Δ^4-pregnen-20β-ol-3-one was available for determination of its infra-red spectrum, but chromato-

graphic mobilities of the two compounds are discrepant (Compound 2 R_F, 0.32; Δ^4-pregnen-20β-ol-3-one R_F, 0.51; in hexane-formamide) (1).

DISCUSSION

Δ^4-Pregnen-20α-ol-3-one was first synthesized by Butenandt and Schmidt (7). Since this steroid and its acetate were both tested and found to be progestationally inactive in the Clauberg test at the 1 mg. level, no further consideration has been given to the compound in subsequent work. To our knowledge this represents the first reported instance of the identification of Δ^4-pregnen-20α-ol-3-one as a metabolite of progesterone. The two compounds identified as major metabolites of progesterone in the human being are pregnan-3α-ol-20-one and pregnane-3α,20α-diol (8, 9). It has been assumed that in man the initial metabolic reactions involved reduction of ring A and occurred in the liver (8). However, Zander and von Münstermann (10) and Butt *et al.* (11) have reported a compound in the plasma of pregnant women which moved with respect to progesterone much like the compound we have described here. It may be that reduction of the 20-carbonyl group occurs in human peripheral tissues as well as in the rat, and that pregnanolone and pregnanediol are synthesized in the liver by reduction of ring A of progesterone and peripherally formed Δ^4-pregnen-20α-ol-3-one, respectively. It must be remembered, however, that the products of progesterone metabolism in the normal rat have not yet been described in the literature, and these do seem to be different from those in the human being.[2]

The technical assistance of Miss Mary Lou Sears is gratefully acknowledged.

SUMMARY

Compound 2, a metabolite of progesterone in eviscerated female rats, has been identified as Δ^4-pregnen-20α-ol-3-one. Its identification is based on a comparison of physical and chemical properties of the free compound and its acetate with those of the authentic compounds. These properties include infra-red spectra, chromatographic behavior, reaction with iodine, and oxidation to progesterone.

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MECHANISM OF ACTION OF 5′-NUCLEOTIDASE*

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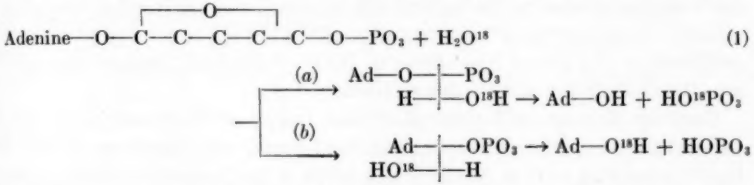
The enzyme 5′-nucleotidase was originally discovered in the venom of snakes (1) and later shown to be present in mammalian tissues (2–5) and bull seminal plasma (6). It has recently been extensively purified and characterized by Heppel and Hilmoe (7). The enzyme was shown to hydrolyze 5′-nucleotides rapidly, ribose-5-phosphate slowly, and other phosphate esters extremely slowly, if at all. Because of its importance in nucleotide metabolism and its interesting specificity pattern, a study of the mechanism of action of the enzyme was initiated.

In order to determine whether carbon-oxygen or phosphorus-oxygen bond rupture occurred during the hydrolytic action, the hydrolysis of adenosine-5-phosphate was followed in H_2O^{18} (see Equation 1). After the cleavage point was determined, exchange experiments of adenosine-C^{14},



$KH_2P^{32}O_4$, and H_2O^{18} with AMP,[1] and of H_2O^{18} with KH_2PO_4, were performed to test the type and life time of the enzyme-substrate intermediates.

EXPERIMENTAL

Hydrolysis of Adenosine-5-phosphate in H_2O^{18}—The enzyme solution (528 units of a purified preparation kindly supplied by Dr. Heppel and Dr. Hilmoe) was added to 50 ml. of H_2O^{18} (about 1.2 atom per cent excess) containing sufficient solid materials to make the final solution 0.04 M in adenylic acid, 0.08 M in glycine, and 0.14 M in magnesium chloride. (Radioactive KH_2PO_4 was added as an analytical control for the H_2O^{18}-AMP exchange experiment described below.) The solution, after adjustment to

* Research carried out at the Brookhaven National Laboratory under the auspices of the United States Atomic Energy Commission.

[1] The following abbreviations were used: AMP for adenosine-5-phosphate, Ad for adenosine, E for enzyme.

XUM

pH 8.5 with solid potassium hydroxide, was allowed to stand for 2 hours at 37°. Samples were then removed for determining the O^{18} content of the medium and the per cent hydrolysis, which was usually about 50 per cent (see below). The inorganic phosphate was precipitated as the barium salt, dissolved in 0.2 N HBr, and reprecipitated three times to remove impurities. The barium phosphate was then converted to KH_2PO_4 with Dowex 50 ion exchange resin (or by sulfate precipitation), isolated, reprecipitated, and pyrolyzed in a manner similar to that described by M. Cohn (8). The O^{18} content of the inorganic phosphate was calculated from the O^{18} content of the CO_2 measured in the mass spectrometer by using a formula similar to the one developed by Dostrovsky and Klein (9).

Exchange Experiment between H_2O^{18} and Adenylic Acid—After removal of the barium phosphate in the hydrolysis experiment described above, the remaining solution was lyophilized. The dry residue was dissolved in ordinary distilled water, and unlabeled phosphate was added and removed as the barium salt. Samples were removed to determine the O^{18} content of the medium and the amount of inorganic phosphate. The adenylic acid present in the supernatant liquid was then hydrolyzed with alkaline phosphatase or 5′-nucleotidase. The phosphate produced in this second hydrolysis was precipitated as the barium salt, purified, and analyzed as described above. Counts of the P^{32} activity were made to correct for any phosphate produced in the initial hydrolysis in H_2O^{18} which had "leaked through" into the unhydrolyzed ester determination.

Exchange Experiment between H_2O^{18} and Inorganic Phosphate—In a typical exchange experiment the enzyme (442 units) was added to 49 ml. of H_2O^{18} containing 0.02 M KH_2PO_4 and 0.008 M tris(hydroxymethyl)aminomethane buffer (pH 8.5). After 29 hours at 37°, more enzyme (442 units) was added, and an aliquot was removed to determine the O^{18} content of the medium. The reaction was stopped after 52 hours by adding barium chloride and removing the phosphate precipitate by centrifugation. The inorganic phosphate was separated and analyzed as described above.

In some of the experiments the enzyme was partially inactivated by heating at 60° or 70° for varying lengths of time. The amount of inactivation was measured by assay under standard conditions after the heat treatment.

Exchange Experiment between Adenosine-C^{14} and Adenylic Acid—Commercial C^{14}-labeled adenosine was purified by chromatographing and rechromatographing with additions of inactive adenylic acid. The purified adenosine-C^{14}, sufficient to give final concentration 0.008 M, was added to a solution containing 9.8 units of enzyme per ml., 0.039 M AMP, 0.08 M glycine, and 0.14 M $MgCl_2$, at pH 8.5. The solution (0.5 ml. total) was incubated for 2 hours at 37° until 48 per cent of the adenylic acid had been hydrolyzed and was then added to the Dowex 1 column. The fractions

were eluted by a modification of the procedures of Cohn and Carter (10). The principal changes were the introduction of larger wash volumes and of inactive carrier adenosine to eliminate contamination of the adenylic acid fraction by adenosine-C^{14}.

Exchange Experiment between $KH_2P^{32}O_4$ and Adenylic Acid—To a solution containing 0.04 M adenylic acid, 0.08 M glycine, 0.16 M $MgCl_2$, and 8.6 units of enzyme per ml. at pH 8.6 was added 0.08 mc. of P^{32} activity as KH_2PO_4. After 2 hours incubation at 37°, the adenylic acid was hydrolyzed to the extent of 42.5 per cent. The inorganic phosphate formed was then eliminated as the barium salt. Inactive inorganic phosphate was added to the supernatant liquid and again precipitated as the barium salt. This procedure was repeated until adenylic acid of constant specific activity was obtained.

TABLE I

Hydrolysis of Adenosine-5-phosphate in H_2O^{18} Catalyzed by 5'-Nucleotidase

The conditions were 0.04 M adenylic acid, 0.08 M glycine, 0.14 M $MgCl_2$, 10.6 units of enzyme per ml.; total volume = 50 ml., pH 8.5, 2 hours at 37°.

Experiment No.	O^{18} atom per cent excess medium	O^{18} atom per cent excess of phosphate produced		
		Observed	Calculated for P—O splitting	Calculated for C—O splitting
1	1.13	0.28	0.28	0
2	1.24	0.28	0.31	0

Results

The results of the hydrolysis and exchange experiments are presented in Tables I to V.

In Table I, the O^{18} content of the inorganic phosphate is seen to agree with the theoretical value calculated for cleavage of the P—O bond during hydrolysis (see Equation 1, *a*). The theoretical value is one-fourth that of the medium because of the symmetrical nature of the phosphate ion and, the presence of the 3 unlabeled oxygen atoms. The slight discrepancy between theoretical and observed values is within experimental error and does not indicate an alternate pathway of hydrolysis.

In Table II are presented the analyses of the unhydrolyzed adenylic acid in the hydrolytic experiments run to about 50 per cent hydrolysis. The observed values must be corrected for the atom per cent excess of the medium (0.014) in which the second (analytical) hydrolysis was performed. Although the H_2O^{18} had been removed by lyophilization, enough water of crystallization remained to give a slight but appreciable added O^{18} content

TABLE II

Exchange of Oxygen between Adenosine-5-phosphate and H_2O^{18} Catalyzed by 5′-Nucleotidase

The conditions were 0.04 M adenylic acid, 0.08 M glycine, 0.14 M $MgCl_2$, 10.6 units of enzyme per ml.; total volume = 50 ml., pH 8.5, 2 hours at 37°.

Experiment No.	Initial hydrolysis in H_2O^{18}		Atom per cent excess of medium during analytical hydrolysis	Observed atom per cent excess of phosphate from AMP	Corrected atom per cent excess in unhydrolyzed AMP
	Per cent of AMP hydrolyzed	Atom per cent excess medium			
1	47.6	1.13	0.014	0.005	+0.001
2	42.5	1.24	0.014	0.001	−0.003

TABLE III

Exchange of Oxygen between H_2O^{18} and KH_2PO_4 Catalyzed by 5′-Nucleotidase

The conditions were 0.02 M KH_2PO_4, 0.008 M tris(hydroxymethyl)aminomethane, pH 8.5; total volume = 52 ml., 52 hours at 37°.

Experiment No.	Total amount of enzyme protein*	Per cent inactivation of enzyme	Atom per cent excess of medium	Atom per cent excess of inorganic phosphate
	units			
1	884	0	1.33	0.021
2	884	85	1.19	0.014
3	1460	10	1.16	0.029
4	1460	98	1.17	0.016
5	730	0	1.23	0.005
6	730	0	1.23	0.009
7	730	99	1.21	0.005
8	730	99	1.21	0.005
9	365	0	1.36	0.004

* Total units present in initial enzyme solution before thermal inactivation. Thus, in Experiment 2 the actual solution added contained 133 active units of enzyme and 751 units of enzyme which had been thermally inactivated.

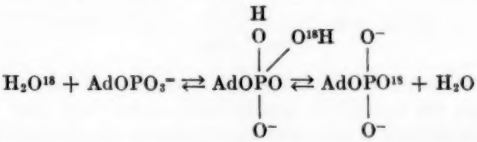
TABLE IV

Exchange between Adenosine-C^{14} and Adenylic Acid Catalyzed by 5′-Nucleotidase

The conditions were 0.039 M adenylic acid, 0.008 M adenosine-C^{14}, 0.08 M glycine, 0.14 M $MgCl_2$, pH 8.5; total volume = 0.5 ml., 2 hours at 37°.

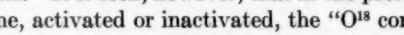
Experiment	Per cent hydrolysis	Per cent C^{14} activity in adenylic acid fraction
No enzyme added		0.02
9.8 units enzyme per ml. added	48	0.05

to the water. The final corrected figure, therefore, is the observed atom per cent excess minus one-fourth that of the medium in the analytical hydrolysis. These values in the last column are clearly zero within experimental error, and hence show that no appreciable exchange of the type seen in Equation 2 is catalyzed by the enzyme.



(2)

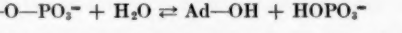
In Table III are some of the experiments designed to test the possibility of exchange of oxygen between water and inorganic phosphate (Equation 3). In Experiments 1 to 4 the experimental error was larger than in the



(3)

subsequent experiments. It is seen, however, that in the presence of varying amounts of enzyme, activated or inactivated, the "O^{18} content" of the phosphate was essentially constant and indicated no exchange. Since this persistent positive blank appeared to be caused by organic impurities, additional purification steps were introduced, and Experiments 5 to 9 confirm the lack of exchange even when the experimental error is one-fifth of the previous value.

In Table IV the experiments on exchange of adenosine-C^{14} and adenylic acid are shown, and it is found that the exchange is less than 0.05 per cent when the hydrolysis has proceeded to about 50 per cent. To obtain the low values illustrated, it was necessary (*a*) to purify the commercially obtained adenosine-C^{14} extensively to remove adenine and adenylic acid impurities and (*b*) to purify the adenylic acid fraction from the column carefully to prevent contamination by small leaks from the adenosine fraction. Previous experiments with less purification had given agreement within experimental error between the enzyme experiment and the control without enzyme but at a higher contamination level (~0.2 per cent). The results with the improved purification show a slight difference between the control and the enzyme run which is of the order of the experimental error.



(4)

A rough calculation of the amount of labeled adenylic acid produced by a reversal of the over-all reaction of Equation 4 gives a figure of 0.016 per cent which is in agreement with the observed difference within experimental error.

The exchange of inorganic phosphate with AMP is shown in Table V.

One possible route for such an exchange is by a reversal of the over-all reaction (Equation 4). A rough calculation indicates that 0.009 per cent of the originally added activity would be added to the AMP by this pathway. The observed result (0.015 per cent) is within experimental error of this value and indicates no alternate avenue for exchange of any importance.

DISCUSSION

The O^{18} experiments clearly establish that the phosphorus-oxygen bond is broken during the hydrolytic action catalyzed by the very specific 5′-nucleotidase. Experiments with O^{18} have already established that the nonspecific acid and alkaline phosphatases (11, 12) and the moderately specific acetyl phosphatase (13) react through phosphorus-oxygen cleavage. Thus,

TABLE V

Exchange of Phosphate between $KH_2P^{32}O_4$ and Adenosine-5-phosphate Catalyzed by 5′-Nucleotidase

The conditions were 0.04 M AMP, 0.08 M glycine, 0.16 M $MgCl_2$, 8.6 units of enzyme per ml., 0.08 mc. of $KH_2P^{32}O_4$, pH 8.6, 2 hours at 37°, 42.5 per cent hydrolyzed.

Purification step	AMP	Calculated per cent of total P^{32} activity in AMP fraction
	c.p.m. per μmole	
1st purification	57.1	1.47
2nd "	1.45	0.037
3rd "	0.52	0.013
Final AMP fraction	0.57	0.015

all the phosphatases examined so far for cleavage point have been found to have P—O splitting, regardless of the specificity pattern of the particular enzyme.

In previous publications evidence has been presented to show that the enzymatic substitution reactions proceed by displacement mechanisms (14, 15). In the case of 5′-nucleotidase, the O^{18} data show that the displacement occurs on the phosphorus atom but do not establish whether the primary attack is made by water, *i.e.* a single displacement mechanism, or enzyme, *i.e.* a double displacement mechanism. (The frontside displacement mechanism can be excluded for phosphate cleavage because of the evidence that the non-enzymatic reactions proceed by bimolecular nucleophilic substitution mechanisms (16).) Morton (17) has shown that the enzyme does not have transferase properties with glycerol as acceptor. Since glycerol is more similar to water than adenosine, it can be concluded that adenosine would be even less likely to substitute for water as an ac-

ceptor. Likewise, according to the specificity for 5′-nucleotides, water cannot replace adenosine at the donor site. Thus exchange of water with KH_2PO_4 or of adenosine with AMP would have to occur via a phosphoryl intermediate, and an observed exchange in either case would establish the mechanism in Equations 5 and 6. Neither was observed.

$$AdOPO_3^- \; EH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} + EPO_3^- + AdOH \tag{5}$$

$$EPO_3^- + H_2O \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EH + HOPO_3^- \tag{6}$$

The results exclude certain alternatives: a stable phosphorylated enzyme intermediate, a rapid reversible first step (Equation 5) followed by a slow second step (Equation 6), or a slow first step followed by a rapid reversible second step. With the currently available data, however, a transient enzyme-phosphate intermediate is still possible.

A single displacement mechanism is also consistent with the data. In this case a direct attack of water on the phosphorus of the adenylic acid would occur in the hydrolysis, and any exchanges of H_2O^{18} with KH_2PO_4 or of adenosine-C^{14} with AMP would require that water could occupy the adenosine site or adenosine occupy the water site. In view of the specificity pattern, neither would be possible (for a discussion of the specificity requirements for exchange, see Koshland (14)), and hence no exchange would be expected as is observed. The lack of exchange of oxygen between H_2O^{18} and the unhydrolyzed AMP, a criterion which has previously been used for hydrolytic reactions (18), indicates that no free addition intermediate of the type $HO^{18}—P(OH)(OH)—OR$ is formed in the reaction. The single displacement, if it is ultimately shown to be the actual mechanism, probably proceeds as in Equation 7 with simultaneous breaking and forming of the bonds indicated by the dotted lines.

$$H_2O + {}^-OPO_3Ad \rightarrow \left[H_2O \cdots P(=O)(O^-) \cdots OAd \right] \rightarrow HOPO_3^- + AdOH \tag{7}$$

The lack of exchange between $KH_2P^{32}O_4$ and adenylic acid is consistent with the previous conclusions. Since a long lived EPO_3 intermediate was excluded, an exchange reaction of the type of Equation 8 would not be expected to compete successfully with the other modes of decomposition

$$EPO_3 + KH_2P^{32}O_4 \rightarrow EP^{32}O_4 + KH_2PO_4 \tag{8}$$

of the intermediate. Similarly, exchange by a single displacement mechanism, *i.e.* by direct attack of phosphate on AMP, would be excluded by the cleavage point evidence and by the specificity pattern of the enzyme. Thus, it can be concluded that the 5′-nucleotidase catalyzes a displacement on phosphorus by either water or enzyme with formation of a transient intermediate.

SUMMARY

1. The point of bond rupture as a result of 5′-nucleotidase action has been studied during the hydrolysis of adenosine-5-phosphate in H_2O^{18}. The presence of a stoichiometric amount of O^{18} in the inorganic phosphate produced is consistent with a displacement mechanism involving an attack on the phosphorus atom.

2. No appreciable exchange was observed on incubating H_2O^{18} with either KH_2PO_4 or adenylic acid. The very small exchanges observed on incubating adenosine-C^{14} with adenylic acid or $KH_2P^{32}O_4$ with adenylic acid agree with the calculations for the amount incorporated by reversal of the over-all reaction and indicate no appreciable exchange by a partial reaction. The exchange experiments establish that the enzyme intermediate must be short lived with respect to decomposition to products.

The authors wish to acknowledge the invaluable assistance of Dr. Lewis Freedman and Mr. John Dencienski with the mass spectrometry.

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RESPIRATORY ENZYMES IN OXIDATIVE PHOSPHORYLATION

VI. THE EFFECTS OF ADENOSINE DIPHOSPHATE ON AZIDE-TREATED MITOCHONDRIA*

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(Received for publication, August 1, 1955)

Papers I to IV of this series (1–4) have dealt with changes in the oxidation-reduction levels of the components of the respiratory chain upon initiation of oxidative phosphorylation in "tightly coupled" preparations of liver mitochondria. Changes in the oxidation-reduction levels of DPNH,[1] flavoprotein, and cytochromes *b* and *c* were readily recorded (3), while those due to cytochromes *a* and a_3 were of small magnitude and difficult to detect. Addition of a low concentration of azide, which does not appreciably inhibit respiration or phosphorylation, greatly magnifies the steady state changes of cytochromes a_3, *a*, and *c* caused by ADP addition. Thus cross-over points (1) in the respiratory chain can be accurately located. It is also possible to "titrate" these components with ADP and to record the speed with which the ADP-cytochrome interaction occurs. Such experiments give insight on the chemical nature of intramitochondrial DPNH and on possible intermediate reaction steps between ADP and the electron transport system.

Preparations—The preparations and reaction media are described in Paper II (2).

Methods—Both the split beam and double beam recording spectrophotometers, as well as the platinum electrode technique, are described briefly in Papers II and I, respectively (2, 3).

Results

Correlation of Oxygen and Cytochrome Kinetics—Whereas the reduction of cytochrome *a* on the transition from State 4 to 3 (see Paper III (1)) causes a very small change in the steady state level of cytochrome *a*, ad-

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[1] The abbreviations used in this paper are ADP = adenosine diphosphate, P_i = inorganic phosphate, ATP = adenosine triphosphate, DPNH = reduced diphosphopyridine nucleotide, DNP = 2,4-dinitrophenol.

dition of azide makes this spectroscopic change much larger, and a typical record obtained in the presence of this inhibitor is given in Fig. 1, *A*. The upper trace records the increased respiration caused by the initiation of oxidative phosphorylation upon ADP addition and its cessation when the added ADP is phosphorylated. The lower trace of Fig. 1, *A* records, as an upward deflection, the reduction of cytochrome *a* during oxidative phosphorylation, followed by its reoxidation when the respiration slackens. It is apparent that the kinetics of the change of the steady state of cytochrome *a* are consistent with the changes of respiratory rate; in fact, the changes in slope of the oxygen trace correspond closely to the amplitude of the changes in the cytochrome *a*.

The spectroscopic changes that accompany the addition of azide to mitochondria in State 4 (Paper III (1)) are illustrated by Fig. 1, *B*: azide addition causes partial reduction of cytochromes *a*, *c*, and a_3, as indicated by the prominent absorption peaks at 605, 550, and 445 mμ. A small shoulder on the long wave-length side of the cytochrome *c* band is caused by a reduction of cytochrome *b* and the trough at 465 to 490 mμ is caused by the reduction of oxidized flavoprotein. If the respiration is increased by the addition of ADP (State 4 to 3 transition), the reduction of cytochromes *a*, *c*, and a_3 increases considerably, as is indicated by the increased heights of their absorption bands. On the other hand, the cytochrome *b* band is less distinct, and that of flavoprotein is definitely smaller, as will be discussed later.

Effect of Azide upon Respiration and Phosphorylation—When a component of the respiratory chain has a high activity relative to the rate-limiting step, its steady state level can be drastically altered without a proportional change of the rate of electron transfer through the chain. This is the case with cytochrome a_3, which reacts with oxygen very rapidly (see Paper IV, Table IV (4)) compared with its turnover number (see Paper II (2)). With glutamate as a substrate as in Fig. 1, *A*, the azide concentration used causes only about 10 per cent inhibition of respiration. If the respiration rate is increased somewhat by using succinate as a substrate, the inhibition is somewhat greater (see Table I). In either case there is a considerable range of azide concentrations within which large changes of the steady state levels of cytochromes *a* and a_3 are observed in the State 4 to 3 transition with only small inhibition of respiration. It should be noted that the respiration in the quiescent State 4 is not measurably inhibited by the higher azide concentrations, while the respiration in the active State 3 is affected (see "Discussion").

Table I also includes some values of the ADP:O ratio in the presence of azide, and it is seen that no decrease occurs up to 276 μM. This result is in accord with that of Loomis and Lipmann, who used a 5-fold greater

azide concentration for the uncoupling of phosphorylation (5). Slater (6) found that about the same azide concentration was necessary, but, in contrast to the results of this paper and of Loomis and Lipmann, found that

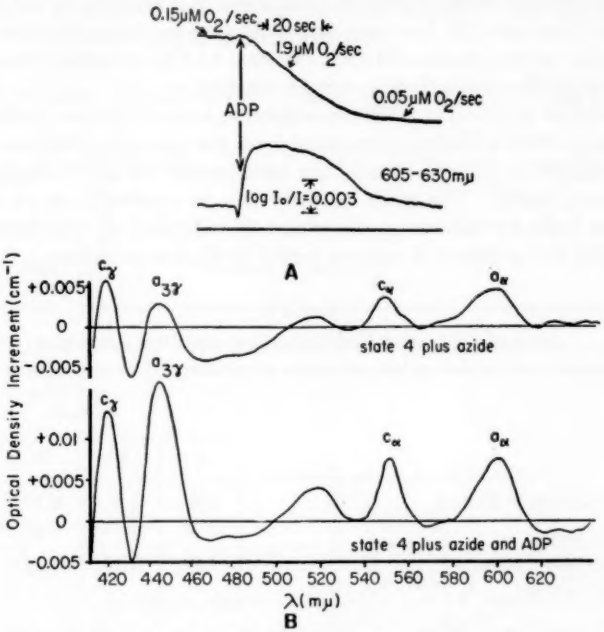

FIG. 1. *A*, correlation between changes in respiration rate caused by ADP addition and changes in the steady state level of cytochrome *a*. The upper trace is recorded by means of a vibrating platinum micro electrode and the lower trace is recorded by means of a double beam spectrophotometer. Guinea pig liver mitochondria, glutamate as substrate, 184 μM azide, isotonic medium, 25° (Experiment 388b). *B*, the effects of azide and azide plus ADP upon the extent of reduction of cytochromes. The base-line in both traces corresponds to mitochondria in State 4; β-hydroxybutyrate as substrate, rat liver mitochondria, 400 μM azide, 1 mM ADP for the lower trace, isotonic medium (Experiment 466f-5).

the respiration of the muscle sarcosomes was much more resistant to azide than that of the liver mitochondria.

Since azide is believed to inhibit respiration by combining with the oxidized form of cytochrome a_3, it might have interfered with the formation of any high energy intermediate attached to the oxidized form of the en-

$$O_2 + a_3'' \times I \rightarrow a_3''' \sim I + O_2^- \qquad (1)$$

zyme and thereby lowered the ADP:O value. Such a decrease is not ob-

served at these azide concentrations and this result is in accord with other data (Paper III (1)) that lead to the conclusion that the reduced and not the oxidized form is bound in the $\sim I$ compound (Paper V (7)). It is also unlikely that the reduced form of cytochrome a_3 is affected by the inhibitor I, since Lehninger (8) has been unable to demonstrate a decrease of the P:O value in the presence of CO. It may be that phosphorylation does not occur in the cytochrome a_3-oxygen reaction.

Affinity for ADP—The ADP concentration required to give maximal respiration in State 3 has been computed from the platinum electrode recordings described in Paper I (3) and has been termed the ADP affinity of the respiratory chain. The same quantity can be evaluated on an entirely different basis by measuring the extent of reduction of cytochrome a in State 3 in the presence of various initial ADP concentrations. A typical

TABLE I

Effect of Azide upon Respiration Rate and Phosphorylation

Guinea pig liver mitochondria, succinate as substrate, 26° (Experiment 378b).

	Azide, μM			
	0	92	276	390
Respiration rate in State 3	2.0	1.9	0.90	0.40
" " " " 4	0.11	0.11	0.26	0.10
Phosphorylative activity $\left(\frac{\text{ADP}}{\text{O}}\right)$	2.0	2.15	2.10	

series of measurements is illustrated by Fig. 2, which shows how both the extent of reduction and the duration of the "cycle" increase with ADP concentration. It should be noted how sensitively this spectroscopic test responds to ADP (see "Discussion").

In a cycle such as that shown in Fig. 2, ADP is acting as a substrate and the simple equation derived for the Michaelis theory may be applied with reasonable accuracy.

$$\frac{p}{e} = \frac{1}{1 + \frac{K_m}{x}} \qquad (2)$$

where p/e is the fractional reduction of cytochrome a, x is the substrate or ADP concentration, and K_m is the affinity.

In Fig. 3, A, we have plotted the increment of reduction of cytochromes a and c as a function of ADP concentration and obtained half maximal effects with about 60 μM ADP for this experiment. A summary of other

data for cytochromes a_3, a, and c is given in Table II. These values are somewhat larger than those computed from the ADP concentration required to give half maximal respiration (20 to 30 μM ADP (3) or 10 μM ADP (9)). It is also possible that the ADP affinity is not identical for the various members of the respiratory chain.

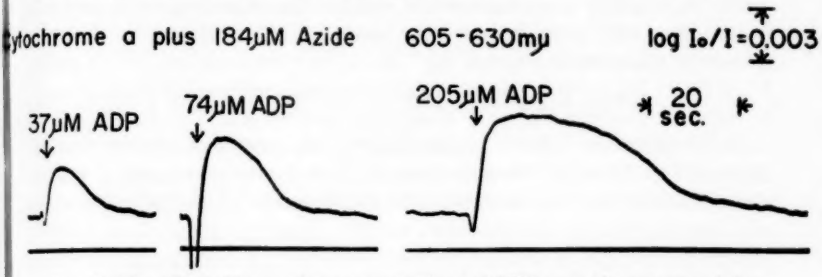

FIG. 2. The effect of increasing concentrations of ADP upon the extent and duration of the reduction of cytochrome a in azide-treated mitochondria. Experimental conditions as in Fig. 1, A.

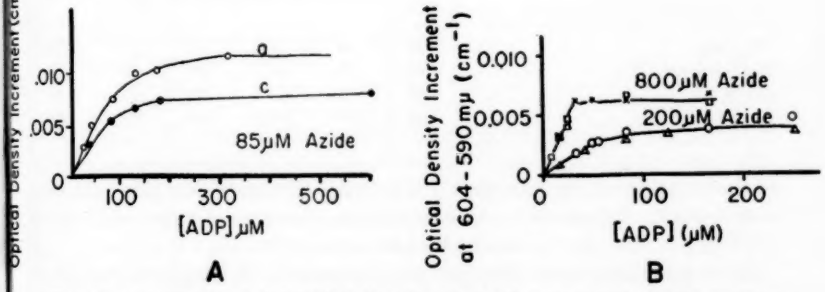

FIG. 3. A, comparison of the ADP titration of cytochromes a and c in the presence of 85 μM azide. Guinea pig liver mitochondria, succinate as substrate, isotonic medium, 10° (Experiment 382b). B, titrations with ADP in the presence of low and high azide concentrations. Rat liver mitochondria, β-hydroxybutyrate as substrate, isotonic medium, 25° (Experiment 459).

A decrease in the rate of electron transport decreases the amount of ADP required for half maximal spectroscopic effects; for example, Table II shows that, in the case of cytochrome a_3, only 23 μM ADP are required at 10°. If excess azide is used, a similar reduction of the ADP concentration is observed (Fig. 3, B), but, under these conditions, the titration curve is no longer hyperbolic (10). At 800 μM azide, the amount of cytochrome a reduced upon ADP addition increases linearly with the ADP concentration

and then breaks sharply at 34 μM ADP, as if a component of the respiratory chain were being titrated.[2] As shown by the spectrophotometric assays of Paper II, Table II (2), the cytochrome *a* concentration is equivalent to about 2 μM, a quantity insufficient to explain this result. In fact, DPNH is the only known component present in sufficient amounts (about 38 μM DPNH in State 4) to account for this result. Thus the simplest hypothesis is that the ADP utilization by the azide-inhibited respiratory chain is caused by the over-all reaction (6)

$$\text{DPNH} \sim I + \text{P}_i + \text{ADP} \longleftrightarrow \text{DPNH} + \text{ATP} + I \qquad (3)$$

As illustrated by Equation 5 (see below), one must also consider that an appreciable amount of the hypothetical $X \sim I$ compound may accumulate in State 4 and may contribute to the titration. The close agreement

TABLE II

Summary of Results on ADP Titrations of Cytochromes a_3, a, and c

Succinate or glutamate as substrate.

	Cytochrome a_3		Cytochrome *a*		Cytochrome *c*	
	Glutamate	Succinate	Glutamate	Succinate	Glutamate	Succinate
"*K*," μM	23	40	44	40	76	~70
Temperature, °C.	10	26	26	26	26	26
Azide, μM	68	85	184	85	184	85
Experiment No.	385	379	388b	379	388	379

of the titration values with the DPNH content suggests that this contribution is small. It has not yet been possible to devise an experiment in which DPNH $\sim I$ and $X \sim I$ can be titrated separately.

If we are observing a titration of a component of the phosphorylation system, then variations in the amount of mitochondria used in the titration should vary the ADP titration value at high azide concentrations. Table III shows that larger amounts of mitochondria require a larger ADP titration to give half maximal effect over the rather narrow range of the experimental data. In no experiments do our titrations fall to the very low value of 10^{-7} M obtained by Slater and Holton for the α-ketoglutarate-cytochrome *c* system (9), and it must be concluded that their value applies only to their preparations of sarcosomes which have much less reducible DPN (11).

[2] The ADP utilization in the 4 second interval required to reach maximal reduction of cytochrome *a* (see Fig. 1) is relatively small. For the conditions of Fig. 3, *B*, ADP utilization due to respiration during the 4 second interval is roughly 8 μM at 34 μM ADP.

Lehninger[3] has observed a DNP-sensitive P^{32} uptake by cyanide-inhibited mitochondria to which ADP is added, and in a particular experiment there was 0.06 mμmole of P uptake per 0.005 mg. of mitochondrial protein, this value being near the maximal one observed in several experiments. In order to compare his result with our titration data, we convert his value to the higher protein concentration used in the experiment of Fig. 3, *B* (4 mg. per cc.). Lehninger's P^{32} uptake is then equivalent to the formation of 48 μM ATP^{32}, as compared with 38 μM DPNH $\sim$ *I* computed to be present from spectrophotometric data, and an end-point of the ADP titration of 34 μM (Fig. 4). This agreement is as good as could be expected in view of the variabilities of mitochondrial preparation. The concordance of these results confirms our rather different experiments (Paper III (1)), on the basis of which we have concluded that mitochondria in State 4 contain DPNH in an inhibited form. These new correlations indicate that the hypothetical compound (DPNH $\sim$ *I*) could convert an equivalent amount

TABLE III

Effect of Mitochondrial Concentrations upon Apparent Affinity of Cytochrome a for ADP

800 μM azide (Experiment 459).

Approximate protein concentration, *mg. per cc.*	2	4	8
ADP for half maximal effect, μM	12	17	22

of ADP to ATP without the need for electron transport. Thus the ADP affinity as obtained from spectroscopic data appears to contain two components: (*a*) a stoichiometric reaction with high energy compounds present in the mitochondria in the resting state and (*b*) a "Michaelis" affinity which depends upon the rate of electron transport in the respiratory chain.

Reaction Kinetics—The kinetics of the State 4 to 3 transition are of considerable importance in evaluating (*a*) whether intermediate chemical reactions or permeability barriers intervene between ADP and its reaction partners and (*b*) whether ADP reacts with the different phosphorylation sites at the same or different speeds.

Table IV shows that the kinetics of oxidation of DPNH and cytochrome *b* upon addition of ADP to the mitochondria in State 4 are not significantly speeded up by an increase in the ADP concentration. Since these two substances are at least three components down the respiratory chain from the terminal oxidase, the maximal speed with which they can be oxidized may be set by the capabilities of the cytochrome chain, and not by the speed of the ADP reaction itself. On the other hand, the speeds of reduction of

[3] Dr. A. L. Lehninger, personal communication.

the cytochromes, especially *c* and *a*, in the azide-treated system are sufficiently rapid to be used for a study of the kinetics of the interaction of ADP with the respiratory chain. As a control experiment, we have compared the speed of reduction of the cytochromes caused by the addition of a sufficient excess of azide (13 mM) to stop respiration rapidly with that caused by the addition of an excess of ADP. The former reaction is found

TABLE IV

Kinetics of State 4 to 3 Transition

Rat liver mitochondria, β-hydroxybutyrate as substrate, 5° (Experiment 306).

Component	ADP, μM			
	96	290	1600	3200
	Half time			
	sec.	*sec.*	*sec.*	*sec.*
Reduced pyridine nucleotide	15	19	26	22
Cytochrome *b*	17	22	17	16

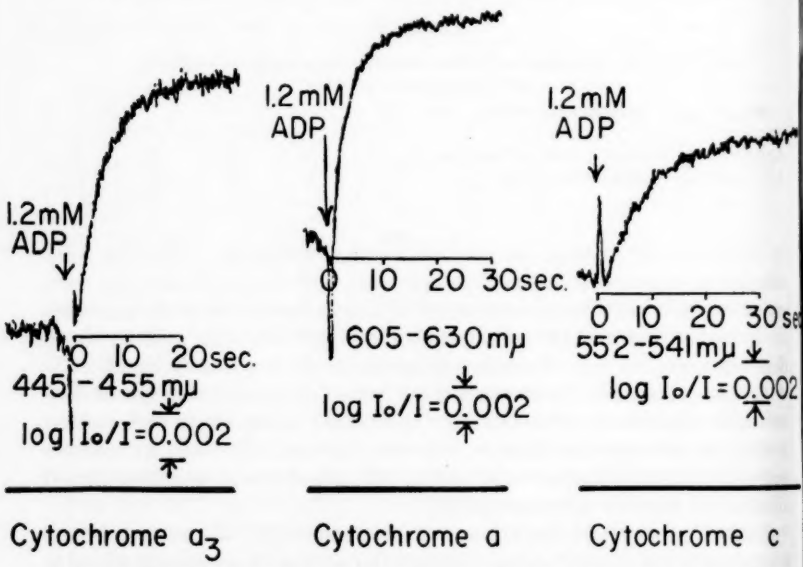

FIG. 4. Direct recordings of the kinetics of cytochrome reduction caused by the addition of ADP to the azide-inhibited mitochondria. Experimental conditions as in Fig. 3, *A*.

to be about 9 times more rapid and this result shows that the kinetics of the latter reaction are not set by the rate of transfer of reducing equivalents along the cytochrome chain, but by the rate of the ADP interaction itself.

Fig. 4 presents direct recordings of the kinetics of reduction of these three cytochrome components measured at appropriate pairs of wavelengths with the double beam spectrophotometer (1). On the basis of half times, the reaction with cytochrome *a* is most rapid. This result is borne out by studies over a wide range of ADP concentrations (see Fig. 5) in which the initial slopes of reaction kinetics, such as those illustrated by Fig. 4, are measured. The initial rate of reaction with cytochrome *a* is more rapid than that with cytochrome *c*, and in both cases the speed of reaction ceases to follow the required linear increase of rate

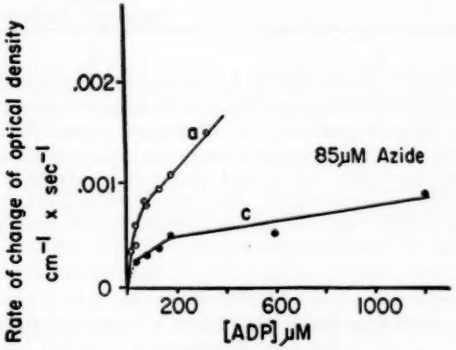

FIG. 5. Rate of reduction of cytochrome *a* or *c* as a function of ADP concentration. Experimental conditions as in Fig. 3, *A*.

with increasing ADP concentration, as if a rate-limiting reaction were interposed between the respiratory chain and ADP. The fact that the kinetics of cytochrome *a* are more rapid than those of cytochrome *c* suggests independently that cytochrome *a* may be bound as cytochrome $a \sim I$ compound.

In order to determine whether this rate limitation is due to a chemical reaction or to impermeability of the mitochondria to added ADP, the reaction kinetics of Fig. 4 have been investigated with hypotonically treated mitochondria. It is found that the kinetics of reduction of cytochrome *a* upon ADP addition to mitochondria that are freely permeable to DPNH (*cf.* Paper III (1)) are no faster than those measured with mitochondria that are impermeable to DPNH. This result is illustrated by the two kinetic traces of Fig. 6, one in isotonic medium, the other in hypotonic medium (0.10 OSM). Thus the break in the kinetic curves of Fig. 5 is due

to one or more chemical reactions interposed between the electron transport chain and the phosphate acceptor. For this reason, the transfer reactions

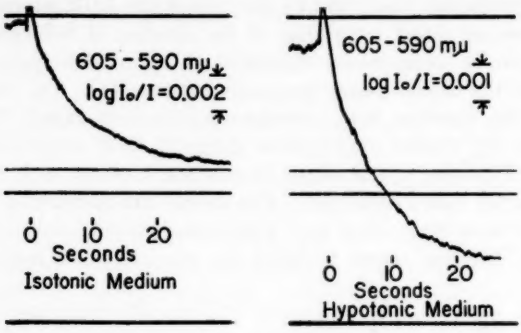

FIG. 6. That the rate of reduction of cytochrome *a* caused by addition of 800 μM ADP to the azide-inhibited mitochondria is not dependent upon the permeability of the mitochondria (Experiment 479a) is illustrated. Note that the sensitivity used for record *B* is about twice that used for record *A* and that the half times of the reactions are about the same. Note that reduction of cytochrome *a* is registered as a downward deflection in this figure. Rat liver mitochondria, succinate as substrate, media, 0.25 and 0.1 OSM, and azide concentrations, respectively, 120 and 40 μM in records *A* and *B*, 10° (Experiment 479a).

TABLE V

Effect of ADP upon Steady State Levels of Azide-Treated Guinea Pig Liver Mitochondria

Succinate as substrate (Experiment 378).

Azide	State No.	Per cent reduction of components					
		a_3	*a*	*c*	*b*	fp	DPNH
μM							
0	4		~0	20	37	46	100
0	3		19	18	18	36	73
92	4	12	19	39	90	71	100
92	3	24	71	54	78	53	69

Heavy rule indicates crossover point. fp = flavoprotein.

might be written as follows, for example, for cytochrome *c:*

$$c'' \sim I + X \longleftrightarrow c'' + X \sim I \quad (4)$$

$$X \sim I + P_i + ATP \longleftrightarrow ADP + X + I \quad (5)$$

Effect of Azide upon Crossover Point—In Paper III (1) we have discussed how the crossover point in the steady state changes caused by the State 3

to 4 transition identifies a pair of respiratory enzymes involved in oxidative phosphorylation. In the uninhibited preparation, the crossover point lies between cytochromes *a* and *c*, as is indicated by the heavy rule in Table V, the preparation consisting of guinea pig liver mitochondria with succinate as a substrate. In the presence of 92 μM azide, the State 3 to 4 transition gives a crossover point further down the chain, between cytochromes *c* and *b*. This effect is also indicated by the spectra of Fig. 1, *B*, as we have pointed out previously. With greater inhibition, it is possible to cause reduction of flavoprotein upon addition of ADP, but still obtain oxidation of DPNH, giving a crossover point between these two components. At even greater inhibition, DPNH and the other components fail to show an oxidation upon addition of ADP. As yet, no crossover point between flavoprotein and cytochrome *b* has been demonstrated.

DISCUSSION

Our finding that respiration in the active state of mitochondria (State 3) is appreciably inhibited by higher azide concentrations (*cf.* Table I) without any appreciable effect upon the resting or State 4 respiration may afford an explanation for the selective effects of azide upon activity and resting respiration, as for example in nerve fibers (12). This effect is in agreement with the hypothesis that phosphate acceptor is generated upon conduction of electrical impulses and accelerates respiration in a small number of mitochondria that are located near the axon surfaces or nodes (13). This activity respiration is azide-sensitive in the manner indicated by Table I, but the azide levels required for nerve are lower than those of Table I (0.1 to 0.2 mM for 90 to 100 per cent inhibition (12)).

The azide-inhibited respiratory chain is a relatively sensitive and specific indicator of ADP. For example, 10^{-5} M ADP gives an optical density change in terms of cytochrome *a* reduction that can be recorded with an accuracy of better than 10 per cent. Thus the sensitivity is of the order of 10^{-6} M in a 1 cc. volume which corresponds to 10^{-9} mole ADP. Interestingly enough, this sensitivity is comparable to that of the firefly luciferase system for ATP (14) when a similar type of photoelectric circuit is used.

It is found that somewhat less ADP is required to give half maximal rate of electron transport, as measured in terms of oxygen consumption, than to give half maximal spectroscopic effects, as measured in the State 4 to 3 transition. It is not unreasonable that this is so, for maximal respiration may be achieved when only the rate-limiting component of the respiratory chain has been saturated with ADP. Other components, present in excess, need not have reacted completely with ADP in order to give maximal rate of electron transport. It has not yet been possible to demonstrate conclusively just what the differences in the titration values for the various

cytochromes may be, although the data of Table II are suggestive. On the other hand, differences of the reaction kinetics of cytochromes *a* and *c* with ADP are clear-cut.

The fact that increasing concentrations of ADP do not cause linearly increasing rates of change of the steady state oxidation-reduction levels of any of the components of the respiratory chain gives direct evidence for intermediates between ADP and the electron transport system. The nature of these intermediates is not known, but several possibilities can be eliminated. For example, similar non-linearities are observed when uncoupling agents such as dibromophenol or dicoumarol is used. Since phosphate is not required for the latter reactions, it follows that these uncoupling agents act in place of ADP + P_i in a reaction similar to that of Equation 5. Thus, in the reaction with an uncoupling agent or with ADP + P_i, the hypothetical $X \sim I$ compound serves as an adequate explanation for the reaction kinetics (15). Other evidence for intermediates in the phosphorylation reaction comes from isotopic studies, but in this case it appears that these intermediates are in the reaction of ADP with phosphate compounds.[4]

SUMMARY

In the presence of appropriate low concentrations of azide as an inhibitor of the respiratory chain of liver mitochondria, the transition from the resting State 4 to the active State 3 caused by addition of ADP shows crossover points (1) between cytochromes *b* and *c* and between flavoprotein and DPNH. These crossover points identify pairs of respiratory enzymes involved in oxidative phosphorylation.

The ADP concentration required for half maximal spectroscopic effects appears to consist of two factors: one related to a Michaelis affinity for ADP and dependent upon the rate of electron transport and the other a titration of an endogenous high energy compound in the resting mitochondria. Since the latter titration value closely approximates the measured content of the intramitochondrial DPNH, it is suggested that DPNH in the resting mitochondria is an inhibited form.

The kinetics of reaction of ADP with the respiratory chain, measured in terms of the speed of the State 4 to 3 transition upon adding ADP, are first order with respect to ADP concentration only for lower values of concentration. At higher concentrations, considerable deviations occur that lead to the conclusion that intermediate reaction steps intervene between ADP and the respiratory chain. In our experiments impermeability to ADP is largely ruled out by observing the same experimental results in hypotonically treated mitochondria.

[4] Dr. Mildred Cohn, personal communication.

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KINETIC STUDIES ON THE RÔLE OF ZINC AND DIPHOSPHOPYRIDINE NUCLEOTIDE IN THE ACTIVITY OF YEAST ALCOHOL DEHYDROGENASE*

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Previous work has demonstrated that the ADH[1] of yeast contains 4 moles of zinc per mole of protein as a structural part of the protein molecule (1–3). The metal has a direct relationship to the catalytic activity of ADH, as shown by (*a*) the concomitant increase of zinc-protein and activity-protein ratios during purification of ADH, (*b*) the inhibition of ADH activity by agents forming stable complexes with zinc, (*c*) the restoration of activity on their removal by dialysis, and (*d*) the prevention or reversal of the action of such inhibitors by Zn^{++}. Mechanisms for the rôle of zinc in ADH activity have been suggested (3). The kinetics of the inhibition by OP, which forms complexes with zinc, and the effect of DPN on this inhibition have been studied to elucidate the participation of the metal in ADH activity. The data indicate that OP competes with DPN for an enzymatically active site of ADH and that each zinc atom of ADH binds a DPN molecule.

EXPERIMENTAL

Twice crystallized yeast ADH was prepared by the method of Racker (4) and stored as a suspension of crystals in 0.60 saturated $(NH_4)_2SO_4$ at 4°. DPN obtained commercially (Pabst Laboratories) was 95 per cent pure as estimated by cyanide complex formation (5). 1,10-Phenanthroline hydrochloride (G. Frederick Smith Chemical Company) was dissolved and titrated with NaOH to the desired pH. The purification of water, chemicals, and glassware has been described (3).

Enzymatic activity was measured by observing the rate of DPNH formation spectrophotometrically at 340 mμ (4), as previously described (3).

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Preliminary accounts of these investigations have been published (19).

[1] Abbreviations: ADH, alcohol dehydrogenase; DPN, diphosphopyridine nucleotide; (DPN), molar concentration of DPN; EtOH, ethanol; (EtOH), molar concentration of EtOH; DPNH, reduced diphosphopyridine nucleotide; OP, 1,10-phenanthroline.

The reaction mixture for the measurement of activity contained 10 γ of twice crystallized yeast ADH in 0.2 ml., measured by the optical density of a fresh stock solution at 280 mμ, based on an absorption coefficient of 0.792 mg. of ADH per sq. cm. (6). The 3.0 ml. reaction mixture contained DPN and ethanol in varying concentrations, as described below, and 1.0 ml. of 0.1 M pyrophosphate buffer, pH 8.8. The reaction rates were measured at 23°. Activity, v, was expressed as change in optical density at 340 mμ per minute per mg. of ADH.

To study the rôle of zinc in the catalytic action of ADH, an inhibitor, OP, was employed to combine with the zinc atoms of [(ADH)Zn_4]. The reaction velocities were measured as a function of the molar concentrations of OP and DPN. Coenzyme and inhibitor concentrations were varied, and resultant data were plotted according to Lineweaver and Burk (7). The lines were fitted to the experimental points by using the equation

$$v^{-1} = a + b\,(\mathrm{DPN})^{-1} \qquad (1)$$

where a and b are constants, $a = V_m^{-1}$, and $b = K_{\mathrm{DPN}} V_m^{-1}$ = regression coefficient.

These lines were determined by the method of least mean squares (8), and only these lines are presented in Figs. 1 to 4. The degree of linearity was expressed as the correlation coefficient (8), $r = (\sigma_{(\mathrm{DPN})^{-1}}/\sigma_{v^{-1}}) \times b$; for data perfectly fitting linearity, $r = 1.000$.

ADH activity is inhibited by zinc complex-forming agents such as OP, when inhibitor and enzyme are in contact with one another prior to initiation of the reaction (3). In these experiments OP was preincubated with the enzyme for 60 minutes at 0°. DPN concentration was varied either in the reaction mixture, providing approximately 5 seconds of contact between DPN and the preincubated ADH-OP mixture before reaction velocity was measured, or in the preincubation mixture, where DPN, ADH, and OP were all in contact for 60 minutes at 0° before activity measurement. Under the latter conditions, the preincubation mixture was prepared by mixing varied amounts of DPN with different OP concentrations and adding ADH in buffer last. The preincubation mixtures were buffered at pH 7.5 or 8.8; the measured pH of the reaction mixture did not change when 0.2 ml. of either preincubation mixture was added. After the mixtures had stood for 60 minutes at 0°, a 0.2 ml. aliquot was removed and added to ethanol in buffer to start the reaction. The reaction velocities were plotted against the final concentration of DPN in the reaction mixture, since it is this concentration which is effective in determining the rate of the enzymatic reaction. The DPN concentration in the preincubation mixture was 15 times greater than that in the reaction mixture. On the other hand, the concentration of OP in the preincubation mixture was used in all calculations

and graphic presentations, since the enzyme-OP complex does not dissociate, and it is this concentration in the preincubation mixture which is the effective one in producing enzymatic inhibition. Since the degree of inhibition by OP is not changed when the preincubation mixture is diluted, the ADH-OP complex is clearly not dissociated upon dilution, and the use of the preincubation concentration of OP is valid.

A suitable concentration of ethanol for these kinetic studies was determined in preliminary experiments under conditions similar to those under which OP inhibition of ADH activity was studied. Five different concentrations of DPN were preincubated with ADH at pH 7.5, the final concentrations in the reaction mixture ranging between 5×10^{-5} and 1×10^{-3} M. Ethanol was not preincubated with ADH, but five different concentrations between 0.267 and 0.400 M were present in the reaction mixture. Lower

TABLE I

Michaelis Constant, K_{DPN}, Estimated at Various (EtOH)

(EtOH)	K_{DPN}	r	S
M	$\times 10^4$ M		$\times 10^4$ M
0.400	6.0	0.963	2.7
0.367	5.5	0.976	2.0
0.333	2.7	0.971	1.8
0.300	6.0	0.994	0.9
0.267	6.4	0.995	0.9

r = correlation coefficient = $(\sigma_{(DPN)^{-1}}/\sigma_{v^{-1}}) \times b$, where b = regression coefficient = $V_m^{-1}K_{DPN}$. S = standard error of estimate = $\sigma_{(DPN)^{-1}}(1 - r^2)^{\frac{1}{2}}$.

or higher concentrations of ethanol resulted in non-linear Lineweaver-Burk plots. The Michaelis constant, K_{DPN}, was calculated from the observed values of v^{-1} and $(DPN)^{-1}$ at each ethanol concentration by the method of least mean squares, and the results are shown in Table I. The correlation coefficient, r, and the standard error of estimate (8), $S = \sigma_{(DPN)^{-1}}(1 - r^2)^{\frac{1}{2}}$, for each set of determinations of K_{DPN} are also presented. The lowest K_{DPN} was found at (EtOH) = 0.333 M, where $K_{DPN} = 2.7 \times 10^{-4}$ M; this concentration of ethanol was therefore used in all experiments in which DPN concentration was rate-limiting.

Results

Effect of DPN on Inhibition of ADH Activity by OP—Fig. 1 shows that DPN partially protects ADH against the inhibitory action of OP, when DPN and ADH are mixed *before* OP is added. On the other hand, the same amount of DPN has no effect on activity when the DPN is added *after*

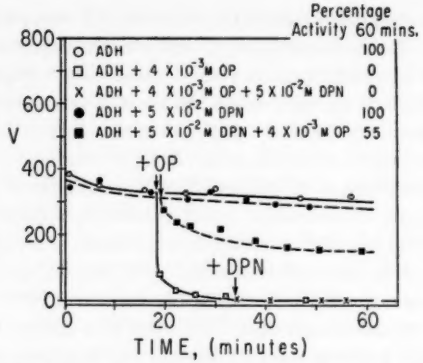
Percentage Activity 60 mins.
○ ADH 100
□ ADH + 4 X 10⁻³ M OP 0
X ADH + 4 X 10⁻³ M OP + 5 X 10⁻² M DPN 0
● ADH + 5 X 10⁻² M DPN 100
■ ADH + 5 X 10⁻² M DPN + 4 X 10⁻³ M OP 55
+OP
+DPN
V
TIME, (minutes)

FIG. 1. Effect of DPN on the inhibition of yeast ADH activity by OP. The solid lines represent rates of the reaction, v, when DPN is not preincubated with ADH; the broken lines represent v when DPN is preincubated with ADH. The addition of OP is marked by arrows, and rates in the presence of OP are shown by □ and ■. The addition of DPN to OP-inhibited enzyme is marked by an arrow and subsequent rates of reaction as ×. Preincubation conditions, ADH ± 5 × 10^{-2} M DPN ± 4 × 10^{-3} M OP, pH 7.5, 0°. For activity measurements, aliquots are removed from each preincubation mixture at the times indicated, and the initial rate of DPN → DPNH is measured at pH 8.8, 23°.

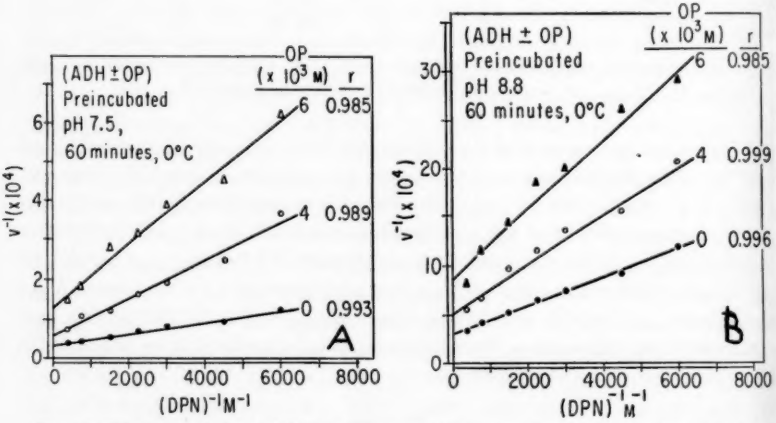

FIG. 2. Kinetics of the inhibition of yeast ADH activity by OP, when DPN is added after ADH and OP are preincubated. All lines are calculated by the method of least mean squares, and r = correlation coefficient. Preincubation conditions, ADH ± varying (OP), 60 minutes, 0°, at (*A*) pH 7.5 or (*B*) pH 8.8. Activity measurements, (DPN) varied in reaction mixture; (EtOH) = 0.33 M; the initial rate of DPN → DPNH is measured at pH 8.8, 23°.

ADH has been mixed with OP and inhibition has occurred; this is also observed at degrees of inhibition less than that shown in Fig. 1.

The quantitative relationships between DPN and OP were examined in the following experiments (see under "Experimental" for the details).

Effect of Varying DPN Concentrations in Reaction Mixture—Fig. 2, *A* and *B*, shows plots of reciprocal reaction velocities *versus* reciprocals of DPN concentrations in the reaction mixture; ADH was preincubated with varying concentrations of OP at pH 7.5 or 8.8. These data fit lines which converge at $v^{-1} = 0$ and $(\text{DPN})^{-1} = -K_{\text{DPN}}{}^{-1}$; the intercepts, $V_m{}^{-1}$, increase

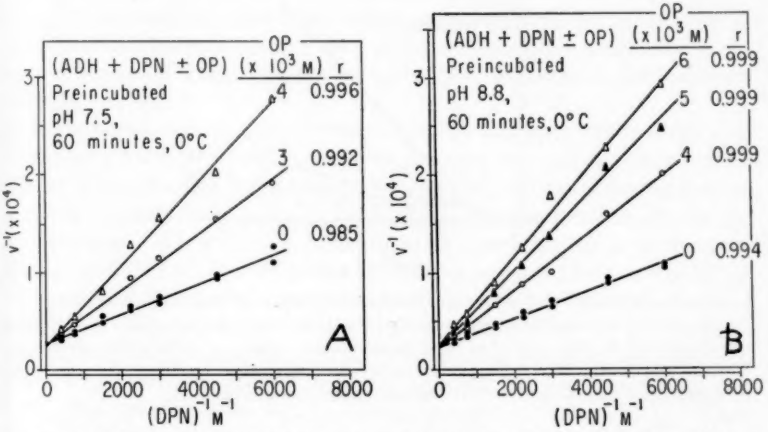

FIG. 3. Kinetics of the inhibition of yeast ADH activity by OP, when DPN is present during the preincubation of ADH and OP. The lines are calculated as in Fig. 2. Preincubation conditions, ADH ± varying (DPN) ± varying (OP), 60 minutes, 0°, at (*A*) pH 7.5 or (*B*) pH 8.8. Activity measurements, (EtOH) = 0.33 M; the initial rate of DPN → DPNH is measured at pH 8.8, 23°.

with increasing OP concentrations. The inhibition of ADH activity by OP is greater when preincubation is performed at pH 7.5 than at 8.8, confirming previous reports (3).

Effect of Varying DPN Concentrations in Preincubation Mixture—Fig. 3, *A* and *B*, demonstrates Lineweaver-Burk plots of v^{-1} *versus* reciprocals of DPN concentrations in the reaction mixture. In contrast to Fig. 2, *A* and *B*, here ADH was preincubated at pH 7.5 or 8.8 with varying concentrations of both OP and DPN. All the calculated lines have a common intercept, corresponding to the extrapolated reciprocal of the maximal uninhibited enzymatic velocity, $V_m{}^{-1}$. Their slopes increase with OP concentration. The inhibition, as above, is greater at pH 7.5.

Fig. 4 demonstrates that the reciprocal reaction velocity, v^{-1}, is a func-

tion of the square of the concentration of OP, the concentration of DPN being constant, when OP, DPN, and ADH are preincubated at pH 7.5. The calculated lines ($r > 0.915$) all converge at $v^{-1} = 3.2 \times 10^{-3}$, $(OP)^2 = -6.5 \times 10^{-6}$ M, when DPN concentration is varied. When OP, DPN, and ADH are preincubated at pH 8.8, similar analysis indicates v^{-1} to be a function of $(OP)^n$, where $2 < n < 3$.

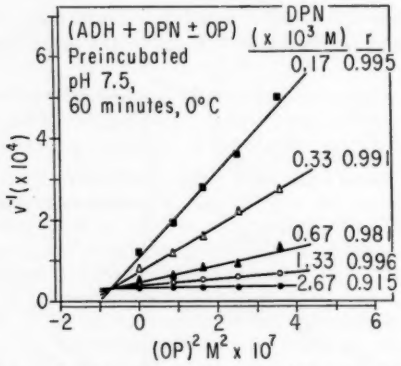

FIG. 4. Dependence of reciprocal reaction velocities of yeast ADH activity, v^{-1}, on the square of the inhibitor concentration, $(OP)^2$, at varying (DPN). All lines are calculated by the method of least mean squares, and r = correlation coefficient. The enzyme-inhibitor complex dissociation constant, K_{OP}, is 6.5×10^{-6} M. Preincubation conditions and activity measurements as in Fig. 3, *A*.

DISCUSSION

The kinetic data, when plotted as reciprocal velocities *versus* reciprocal coenzyme concentrations, fit straight lines both in the presence and absence of inhibition of the enzyme. The linearity is statistically significant over a 16-fold range of coenzyme concentrations, in spite of the increase in experimental error inherent in the measurement of inhibited reaction rates and in spite of the further accentuation of apparent error when reciprocal reaction velocities are plotted (9). The assumptions of Michaelis and Menten (10) are therefore applicable when DPN is present in rate-limiting concentrations, as has, indeed, been demonstrated elsewhere for yeast ADH in the presence of high ethanol concentrations (6).

1,10-Phenanthroline is particularly suitable as an inhibitor in these studies on the rôle of the metal in yeast ADH, because it forms stable zinc complexes (11, 12). All evidence thus far (3) is consistent with the inference that this inhibitor combines with a zinc atom of $[(ADH)Zn_4]$. However, if the mechanism of inhibition of ADH is to be interpreted as the formation of a Zn-OP complex, it must be pointed out that the interaction of OP with Zn^{++} ions in solution differs from that with the zinc atoms of

[(ADH)Zn_4] in at least one important respect, when OP is preincubated with enzyme in the absence of ethanol. The formation of complexes of OP and Zn^{++} ions is instantaneous (11, 12), but OP in contact with [(ADH)Zn_4] inhibits activity at a slow and measurable rate (3) when OP is preincubated with the enzyme alone. The linearity of the reciprocal enzyme-velocity plots implies that an inactive enzyme-inhibitor complex *does* form. At present, there is no experimental evidence to explain the slow rate of formation of this [(ADH)Zn_4]-OP complex. The firm binding of the zinc atoms to the apoenzyme of ADH constitutes obvious differences from systems in which Zn^{++} ions are in solution and makes analogies difficult. It can only be speculated that changes either in the entire protein, or in the ligand groups binding zinc, slowly increase the accessibility of the zinc atoms to OP. If such changes occur, they are at least partly reversible (3).

Steric factors which probably influence the binding of OP to the zinc atoms of [(ADH)Zn_4] may also affect the enzyme-coenzyme interaction. The sequence of exposure of ADH to OP and DPN is critical for the demonstration of competition between the inhibitor and the coenzyme for an enzymatic site. When DPN is added to ADH *before* (Fig. 1), or at the *same time* (Fig. 3, *A* and *B*), incubation with OP is started, the presence of the coenzyme influences the degree of inhibition. Under these experimental conditions, reciprocal velocity-concentration plots indicate strictly competitive inhibition (7, 9, 13) between the zinc-binding inhibitor and the coenzyme. However, when DPN is added *after* OP has been incubated with ADH, the coenzyme does *not* affect the degree of inhibition (Fig. 1). Variation of DPN concentration under these experimental conditions, *i.e.* in the reaction mixture, produces inhibition that is not competitive between inhibitor and coenzyme (Fig. 2). The decrease in activity, although meeting some criteria for "non-competitive" inhibition (13), does not conform exactly to other (9) criteria for "strict" non-competitive or uncompetitive inhibition. The coenzyme apparently does not *displace* OP from the already formed ADH-OP complex, although it seems to *compete* with OP for an unoccupied (or sterically unaltered) enzymatic site.

The kinetic data (Fig. 4) are consistent with the competition of 2 molecules of OP for each enzymatic site (14) when OP, DPN, and ADH are preincubated at pH 7.5. The reciprocal velocity plots converge at a point which indicates the dissociation constant of the enzyme-inhibitor complex (13): $K_{OP} = 6.5 \times 10^{-6}$ M. Previous studies (3) on the activity of ADH preincubated with varying concentrations of OP at pH 7.5 indicate that, when 2 molecules of OP act on one enzymatic site, $K_{OP} = 5.8 \times 10^{-6}$ M. The similarity between the two dissociation constants calculated from different types of experiments supports the interpretation of the kinetic data. The reaction which forms the inactive enzyme-inhibitor complex at pH

7.5 may thus be formulated on the basis of experimental data (3) and considerations previously discussed (15).

$$[(ADH)Zn_4] + 4(2OP) \rightleftarrows [(ADH)Zn_4](2OP)_4 \qquad (2)$$

Structural and kinetic investigations on zinc metalloenzymes have recently been reviewed in detail, and the use of empirical formulae for metalloenzymes has been discussed (15). In extension of this work, the studies reported here were designed to elucidate, by the use of a zinc-binding inhibitor of ADH activity, the rôle of zinc in binding DPN to $[(ADH)Zn_4]$. It seems possible to draw certain inferences from experiments, such as that shown in Fig. 4, concerning the binding of Zn to the yeast ADH apoenzyme. Many observations indicate that zinc has a possible coordination number of six, as for instance, in forming chelate complexes with OP (11). Other ligands form zinc complexes in which the metal has a maximal coordination number of four, *e.g.* in the coordinated complexes with imidazole (16). No evidence is available at present to indicate how many of the six possible coordination bonds of each zinc atom in $[(ADH)Zn_4]$ are occupied by the ligand groups of the apoenzyme (ADH). The apoenzyme probably ligates at least two metal bonds: OP forms the complex $[(ADH)Zn_4](2OP)_4$ in which four bonds of each metal atom are occupied by OP, and the zinc is still bound to the protein presumably through its two remaining bonds. Thus a hexacoordinate zinc atom must exist *under these circumstances*. It is possible that OP *forces* a maximal coordination number of six on the enzyme-bound zinc atoms, where zinc *may* usually have only four. Therefore, these data neither resolve the mode of binding of zinc to the ADH molecule nor the coordination number of zinc in the "natural state" of this enzyme. Further experimentation, however, should clarify these questions.

Comparison of analytical evidence (3) with the stoichiometry of DPN-binding to yeast ADH suggests that zinc is a site of binding of DPN, since 4 molecules of DPN are bound per molecule of protein (6). This is consistent with evidence that OP inhibits ADH activity by forming a complex with the zinc of $[(ADH)Zn_4]$ and that OP competes with DPN. However, analysis of kinetic data indicates only the *possibility* of a common site of action of DPN and OP, but does not *prove* it as a unique solution. As emphasized by Ogston (17), deductions on the chemical identity of enzymatic sites of combinations cannot and should not be drawn from kinetic studies alone.

However, from the evidence discussed above, it is concluded that 1 molecule of DPN is bound to each zinc atom of $[(ADH)Zn_4]$ to form the active enzyme-coenzyme complex $[(ADH)Zn_4](DPN)_4$. As previously empha-

sized (3), these findings do not rule out the existence of other linkages between DPN and yeast ADH, such as sulfhydryl groups (18), in addition to zinc.

The interpretations presented are compatible with the known characteristics of zinc chelates with other organic molecules of structures simpler than is that of ADH.

SUMMARY

The kinetics of the interaction between DPN and 1,10-phenanthroline, a zinc-binding inhibitor of ADH activity, were studied to elucidate the mechanism of action of yeast [(ADH)Zn_4]. Inhibition of ADH activity is competitive between OP and DPN. When DPN is added to ADH before OP, inhibition of activity is decreased, indicating protection of the enzyme against the action of OP. When DPN is added to ADH after OP, activity is not changed, indicating that there is no restoration of activity in the OP-inhibited enzyme. 2 molecules of OP seem to bind to 1 zinc atom of [(ADH)Zn_4] to form the inactive enzyme-inhibitor complex [(ADH)Zn_4]-$(2OP)_4$; the apparent dissociation constant, K_{OP}, is 6.5×10^{-6} M. The data indicate that 1 molecule of DPN binds to each atom of zinc of yeast [(ADH)Zn_4] to form the active enzyme-coenzyme complex [(ADH)Zn_4]-$(DPN)_4$.

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THE EFFECT OF FOLIC ACID AND VITAMIN B_{12} ON THE SYNTHESIS OF SERINE AND CHOLINE FROM GLYCINE IN THE LIVER OF YOUNG TURKEY POULTS

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The incorporation of the nitrogen of glycine and serine into choline was observed by Stetten (1). Later work showed that in the normal rat glycine and serine can be used for synthesis of the ethanolamine moiety and methyl groups of choline (2, 3). Glycine is converted first to serine and then by decarboxylation to ethanolamine which is available to form that moiety of choline. The β-carbon of serine is converted to a methyl group without the loss of the 2 hydrogen atoms during the reaction (4). It is thus possible for the α-carbon of glycine to appear in any of the carbon positions of choline during normal metabolism.

It has been clearly demonstrated that vitamin B_{12} is necessary in some manner for the synthesis of the labile methyl groups (5–8). In a deficiency of vitamin B_{12}, less synthesis of methyl groups of choline takes place from the α-carbon of glycine in the rat (9, 10). In the pig (11) a deficiency of vitamin B_{12} reduced the synthesis of the methyl groups of choline but did not influence the synthesis of serine or ethanolamine. Supplementing a vitamin B_{12}-deficient diet with vitamin B_{12} increased the ability of the liver of chicks to convert folic acid to folinic acid *in vitro* (12).

The exact function of vitamin B_{12} in the synthesis of the methyl group from the α-carbon of glycine is not apparent. Arnstein (13) has proposed that it is needed for the reduction of a hydroxymethyl derivative of folic acid to a methyl group.

In turkey poults vitamin B_{12} is presumed to be required for the synthesis of methyl groups (14), since deficient poults grow slowly on a deficient ration containing homocystine and show improved growth with the addition of vitamin B_{12}. Betaine also caused growth improvement, indicating that transmethylation was not impaired by the deficiency.

A deficiency of folic acid has been found to reduce the use of the β-carbon of serine, and, to a smaller extent, the α-carbon of glycine for the synthesis of choline (15, 16). Tetrahydrofolic acid (THFA) is presumably the active form of folic acid in these reactions. Folic acid is required by the poults (17, 18) but has not been studied in relation to choline synthesis.

The present experiment attempts to determine the rôle of vitamin B_{12} and folic acid in choline synthesis from glycine-2-C^{14} in the turkey poult.

EXPERIMENTAL

Poults hatched from turkey hens deficient in folic acid[1] were fed a ration deficient in folic acid. The ration contained acetone-extracted fish-meal 38 per cent, cellulose 5.0 per cent, soy bean oil 3.5 per cent, and dimethylethanolamine, minerals, choline, vitamins[2] other than folic acid, and corn-starch to equal 100 per cent. High mortality and poor growth were noted in the poults fed this ration, while satisfactory results were obtained when it was supplemented with 10 mg. of folic acid per kilo.

Poults hatched from turkey hens deficient in vitamin B_{12} (19) were fed a ration deficient in vitamin B_{12}. The vitamin B_{12}-deficient diet contained isolated soy bean protein (Drackett C-1) 30 per cent, calcium gluconate 5.0 per cent, soy bean oil 3.5 per cent, dimethylethanolamine 0.111 per cent, DL-methionine 0.2 per cent, homocystine 0.25 per cent, glycine 0.5 per cent, minerals, vitamins except vitamin B_{12} and choline, and corn-starch to equal 100 per cent. The vitamin B_{12}-deficient poults grew more slowly than those supplemented with the vitamin.

After the poults were approximately 3 weeks old, one bird each from the folic acid-supplemented, folic acid-deficient, and vitamin B_{12}-deficient groups was sacrificed and its liver removed immediately. With a modified Krebs-Henseleit buffer (20), the liver was homogenized at 6° in a Potter-Elvehjem homogenizer. 4 gm. of liver were treated with 8 ml. of buffer, and another 0.5 ml. of the ice-cold buffer was used to wash the homogenizer. Liver homogenates were prepared from all of the three birds in this way.

The radioactive glycine labeled in carbon 2, used in the present study, had a specific activity of 0.9 mc. per mmole, and was obtained from The Bio-Rad Laboratories, Berkeley, California. 0.1 mc. of this glycine was dissolved in 3.5 ml. of distilled water.

1 ml. of a solution of $NH_2C^{14}H_2COOH$ containing 28.6 μc. of activity was added to 8.0 ml. of liver homogenate. The mixture was incubated in a stoppered Erlenmeyer flask for 2 hours at 38° with continuous shaking. The stoppers were opened two times during the incubation. The mixture was frozen immediately after the incubation period.

Extraction of Phospholipides—To the flask incubated with normal liver homogenate, 5 ml. of 95 per cent ethanol were added, and the flask was kept at 60° for 30 minutes. Then the contents were transferred to centrifuge tubes and spun for 5 minutes in an angle head centrifuge. The turbid supernatant fluid was transferred to a 100 ml. beaker. The original flask

[1] Kratzer, F. H., unpublished observations.

[2] We are indebted to Merck and Company, Inc., Rahway, New Jersey the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, and The Dow Chemical Company, Midland, Michigan, for kindly donating vitamins and amino acids used in this work.

was washed with 5 ml. of absolute alcohol and the alcohol was then transferred to the centrifuge tubes. It was kept at 60° for 10 minutes and then respun for 3 minutes. The supernatant fraction was transferred again to the beaker. This process was repeated five times so that the total volume of supernatant fluid was about 30 ml. It was finally extracted with acetone.

The solvents were evaporated on a steam bath, and the residue was extracted three times with 10 ml. portions of petroleum ether. A fourth extraction was made with dry acetone and the extracts were transferred to an Erlenmeyer flask and evaporated on a steam bath.

Hydrolysis of Phospholipides to Yield Choline—15 ml. of 33 per cent vol-lume per volume HCl were added to the flask and refluxed for 1½ hours. The solution was extracted three times with 5 ml. portions of petroleum ether, and then the acid solution was evaporated to dryness under an infrared lamp. The dried mass was dissolved in distilled water, and the volume was made to 25 ml. In this way, extracts were prepared from the other samples also; namely, folic acid-deficient and vitamin B_{12}-deficient livers. By using the method of Horowitz and Beadle (21), the choline content of these extracts was determined microbiologically.

To each sample, 130.2 mg. of choline were added, and the solutions were evaporated to dryness under the heat lamp. The choline was isolated from each sample as chloroplatinic acid complex by the method of du Vigneaud *et al.* (22). The choline chloroplatinate was crystallized to a constant specific activity from 80 per cent alcohol and the platinum content determined microanalytically. Furthermore, the choline chloroplatinate was degraded to yield trimethylamine chloroplatinate (22), and the specific activity of the trimethylamine moiety was determined. From this, the per cent incorporation of radiocarbon in the trimethylamine moiety was calculated as compared to the specific radioactivity of the choline molecule.

Isolation of Serine and Glycine—The total solids, after isolation of phospholipides, were pooled together and hydrolyzed with 15 ml. of 6 N HCl in a sealed glass tube for about 16 hours at 15 pounds pressure. The hydrolysates were evaporated to dryness, extracted with water, decolorized with charcoal, and made to 25 ml. By using 2 ml. of the hydrolysate, glycine and serine were isolated on a Dowex 50 column with 1.5 N HCl by the method of Stein and Moore (23). The glycine and serine content was determined colorimetrically with ninhydrin, according to Moore and Stein (24).

Each serine sample was diluted with 157 mg. of inactive serine and crystallized to constant activity. The specific activity of serine was determined, and it was, further, degraded by the method of Sakami (25) to determine the per cent activity in each carbon.

All carbon radioactivity determinations were carried out by wet combustion (26) to CO_2, and the carbon was precipitated as $BaCO_3$ and counted in a gas flow counter.

RESULTS AND DISCUSSION

In the liver of the normal poult there was a considerable synthesis of serine and choline from the α-carbon of glycine (Table I). With a deficiency of folic acid there was a marked reduction in the synthesis of serine, and choline synthesis was slightly reduced. A deficiency of vitamin B_{12} caused no reduction in serine synthesis, but there was a slight reduction in the synthesis of liver choline. The livers of folic acid-deficient poults utilized less of α-carbon of glycine than did those of the normal or vitamin B_{12}-deficient birds.

TABLE I

*Activity of Choline, Serine, and Glycine after Incubating Turkey Liver Homogenates with Glycine-2-C^{14}**

Type of liver	Activity, c.p.m. per mole		
	Choline × 10^5	Serine × 10^5	Glycine × 10^5
Normal	8.43	20.6	29
Folic acid-deficient	5.88	4.65	77.5
Vitamin B_{12}-deficient	5.92	22.7	10

* 28.6 μc. per flask.

TABLE II

Distribution of C^{14} in Choline and Serine after Incubation of Turkey Liver Homogenates with Glycine-2-C^{14}

Type of liver	Choline $N(CH_3)_3$	Serine		
		C_1	C_2	C_3
	per cent	*per cent*	*per cent*	*per cent*
Normal	14.9	0.5	59.5	40.0
Folic acid-deficient	26.9	0.5	67.5	32.2
Vitamin B_{12}-deficient	15.4	0.2	80.6	19.2

Although the amount of choline synthesized by the liver of the folic acid-deficient poult was slightly reduced, the amount of C^{14} in the methyl group (Table II) was considerably increased, compared with that of the control bird. This indicated a slight increase in total methyl group synthesis. The distribution of C^{14} in the methyl and ethanolamine moieties of choline for the vitamin B_{12}-deficient poult was similar to that of the normal bird.

There was very little activity in the carboxyl carbon of serine in the samples from any of the birds. The liver of the folic acid-deficient bird incorporated slightly less C^{14} in the 3 position of serine and more in the 2 position. In the liver of the vitamin B_{12}-deficient poult the amount of C^{14} in the 3 position was approximately half that of the control, with a corresponding increase in the amount in the 2 position.

The proposed mechanism for the incorporation of C^{14} from the α-carbon of glycine to choline (Fig. 1) is based largely on those proposed by Elwyn

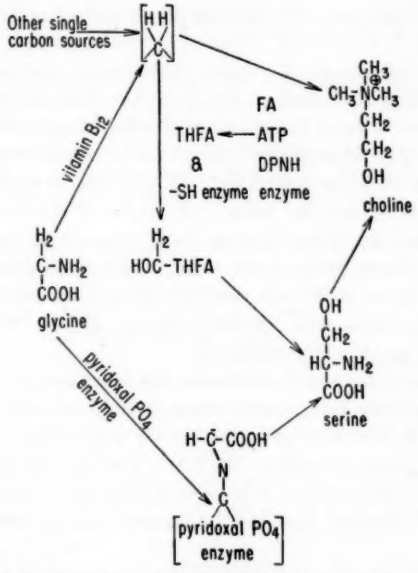

FIG. 1. Mechanism for the incorporation of C^{14} from the α-carbon of glycine into choline.

et al. (4) and Alexander and Greenberg (16). In the normal animal, glycine may conjugate with pyridoxal phosphate and an enzyme, which in turn may react with hydroxymethyl THFA to form serine. By an alternate route glycine may also be used as a source of the hydroxymethyl portion of the hydroxymethyl THFA. Thus, the α-carbon of glycine may appear in both the 2 and 3 positions of serine. Serine can be decarboxylated to form ethanolamine, which in turn can be used in choline synthesis. The methyl groups of choline may also be derived from the α-carbon of glycine. Thus, it is possible that the α-carbon of glycine may appear in any of the positions in choline.

In a deficiency of folic acid, there is a reduction in the THFA which results in a reduction of the amount of available hydroxymethyl THFA, and hence a marked reduction in serine synthesis. The fact that there was a greater reduction in the number 3 carbon atom of serine than in the 2 position indicates that the total serine reduction was the result of a limited availability of hydroxymethyl THFA. The reduced serine probably limited the synthesis of choline since the amount of ethanolamine moiety was reduced in the choline. The increased proportion of C^{14} in the methyl groups of choline shows that methyl synthesis was not reduced and indicates that methyl synthesis probably did not proceed through a folic acid derivative.

In a deficiency of vitamin B_{12} there was no reduction in total serine, but there was a marked reduction in the 3 carbon derived from the α-carbon of glycine. Thus, vitamin B_{12} is not involved in the synthesis of the 2 position through the pyridoxal-phosphate enzyme complex but is necessary for the incorporation into the 3 position. This could be explained by the necessity of vitamin B_{12} for the initial conversion of the α-carbon of glycine to a single carbon compound before its incorporation into the hydroxymethyl THFA. Presumably, some other single carbon sources must have been available, since there was no reduction in total serine synthesis. Methyl group synthesis was reduced slightly, due to the fact that total choline synthesis was slightly reduced.

This mechanism proposes that vitamin B_{12} functions in the conversion of the α-carbon of glycine to a single carbon compound and differs from that of Arnstein (13) in which he proposes that vitamin B_{12} is essential for the reduction of the hydroxymethyl THFA. The fact that a deficiency of folic acid did not limit methyl synthesis is the main argument in support of the function of vitamin B_{12} early in the conversion from glycine.

SUMMARY

1. Livers from normal, folic acid-deficient, and vitamin B_{12}-deficient turkey poults were incubated with glycine-2-C^{14}.

2. In the normal poult the α-carbon of glycine was found in the 2 and 3 positions of serine, and in both the methyl groups and ethanolamine moiety of choline.

3. A folic acid deficiency reduced the total amount of serine synthesized from the α-carbon of glycine with a greater reduction in the incorporation of C^{14} in the 3 position than in the 2 carbon. Choline synthesis was reduced slightly with a reduction of incorporation of C^{14} in the ethanolamine moiety and an increase in the methyl groups.

4. A deficiency of vitamin B_{12} did not reduce total serine synthesis but reduced the incorporation of C^{14} into the 3 position. Choline synthesis was

reduced slightly with the reduction in incorporation in the methyl group proportional to that of total choline.

5. The data suggest that vitamin B_{12} functions in the conversion of the α-carbon of glycine to a single carbon unit which can be used both for methyl synthesis and for conversion to hydroxymethyl THFA.

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EFFECT OF GLUCOSE ON FATTY ACID AND GLYCERIDE SYNTHESIS IN RAT MAMMARY GLAND*

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It has been repeatedly demonstrated that the extent of lipogenesis occurring in tissues like liver and mammary gland is related to carbohydrate utilization by those tissues (1–6). Folley and French (7) have suggested three possible functions of the carbohydrate: (1) it may provide a source of energy required in fatty acid synthesis; (2) it may provide some of the carbon required for the fatty acid synthesis; and (3) it may provide glycerol for the esterification of the fatty acids. Balmain *et al.* (8) found that glycerol is not the equal of glucose in stimulating lipogenesis from acetate in rat mammary gland slices, and therefore they concluded that availability of glycerol is not the complete explanation of the glucose effect on lipogenesis. We have shown, however, that *substrate* glycerol is not utilized to any great extent by rat mammary gland (3). Since glycerol formed via glycolysis may differ in reactivity from substrate glycerol, it is conceivable that experiments carried out with added glycerol may not test whether glycerol formation from glucose is a limiting factor in fatty acid formation from acetate.

The above investigations were concerned largely with the action of glucose on the 2-carbon unit. Since triglycerides containing short chain fatty acids are normal constituents of milk, we studied the conversion by the mammary gland of C^{14}-labeled butyrate, hexanoate, and octanoate, as well as acetate, to glycerides. It is shown here that glucose does promote the incorporation of the C^{14} of hexanoate-1-C^{14}, butyrate-1-C^{14}, and acetate-1-C^{14} into glyceride fatty acids with chain lengths of 8 carbons or more. In the case of octanoate and hexanoate, the glucose promoted their direct esterification, and this appears to be the sole action of glucose upon octanoate. Thus, one of the effects of glucose in promoting lipogenesis results from its action upon fatty acid esterification.

EXPERIMENTAL

Rats of the Long-Evans strain were fed a stock diet with the following composition: 67.5 per cent whole wheat; 15 per cent casein; 7.5 per cent

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skim milk powder; 6.75 per cent hydrogenated vegetable oil; 1.5 per cent $CaCO_3$; 0.75 per cent NaCl (containing a trace of KI); and 1 per cent fish-oil concentrate containing 3000 U.S.P. units of vitamin A and 400 international chick units of vitamin D per gm.

Substrates

Acetate-1-C^{14}, butyrate-1-C^{14}, hexanoate-1-C^{14}, and octanoate-1-C^{14} were prepared by carboxylation with $C^{14}O_2$ of the appropriate Grignard reagent. They were incubated as the sodium salt. We are indebted to Dr. S. Abraham for their preparation.

Preparation of Slices and Incubation Procedure

Lactating rats were sacrificed by a sharp blow on the head, and the mammary glands were removed rapidly and placed in cold, oxygenated bicarbonate buffer (9). Slices were prepared with an automatic slicing machine purchased from H. Mickle (10). The slices were washed in three fresh portions of buffer solution to deplete the tissue of preformed milk, gently blotted on filter paper, weighed on a torsion balance, and transferred to an incubation flask. The incubation procedure has been described elsewhere (3, 11). The Krebs-Henseleit bicarbonate buffer was prepared free of calcium ion to avoid precipitation of the short chain fatty acids.

Analytical Procedures

Collection and Determination of $C^{14}O_2$ Evolved—The incubation flask was designed to permit the collection of CO_2 at the end of the experiment. The determination of the C^{14} content of this CO_2 has been described elsewhere (12).

Extraction of Total Lipides—The acidified incubation medium was decanted through glass wool, and the tissue residue was thoroughly washed with water. The filtrate was discarded. The tissue and the glass wool were extracted four times with a mixture of 3 parts ethyl alcohol and 1 part ethyl ether. To aid the extraction, the mixture was heated each time on a steam bath for 15 minutes.

Separation of Neutral Lipide-C^{14} from Added C^{14} Fatty Acids—Since we were interested in the portion of *added* labeled fatty acids that had become esterified, it was necessary to separate the free, labeled fatty acids, added to the medium as Na salts, from the neutral lipides. This was done as described by Cason *et al.* (13). The combined alcohol-ether extracts were evaporated almost to dryness on a steam bath. A small amount of water was then added to the residue, and the lipides were extracted with ethyl ether. The free fatty acids were removed by passing the ether solution over a small ion exchange column (Amberlite IRA-400) which had been

previously charged with NaOH. The column contained approximately 1 gm. of resin, which is sufficient to remove 100 mg. of palmitic acid or its equivalent. The column was washed four times with 20 ml. of ethyl ether, and these washings, which contained the neutral lipides, were combined. An aliquot of this ether extract was hydrolyzed with sodium ethylate as described elsewhere (11). A small amount (4 ml.) of ethyl ether was added to the hydrolysate, and the mixture was acidified and kept cool to avoid loss of the short chain fatty acids. The cold, acidified solution was extracted several times with a large volume of ethyl ether, and the ether extracts were combined.

Chromatographic Separation of Short Chain Fatty Acids—An aliquot of the ethyl ether extract of the fatty acid was steam-distilled, and the distillate was collected in excess base. The distillate was concentrated and acidified, and the free fatty acids were extracted with isooctane. The precautions mentioned above were again employed to avoid loss of the volatile fatty acids. Carrier fatty acids with chain lengths of 4, 6, 8, and 10 carbons were added to the isooctane extract and separated on a silicic acid column according to the method of Ramsey and Patterson (14). The method was standardized by separating solutions containing various mixtures of short chain fatty acids, only one of which was labeled with C^{14}, and determining the C^{14} content and titration values of the separate fractions. By plotting these values, it was established that a quantitative recovery of the C^{14} was obtained within the titration curve of a particular labeled fatty acid.

The residue in the steam distillation flask was repeatedly extracted with ethyl ether. The ethyl ether extracts were combined. This fraction contained the non-volatile fatty acids.

Lipide-C^{14} Determination—The C^{14} content of the neutral lipide fraction was determined by the direct mount technique described by Entenman *et al.* (12). Fatty acid-C^{14} was determined by mounting an aliquot of the fatty acids on lens paper, with corn oil as carrier (12). No heat was used in mounting these fatty acids. The C^{14} content of the individual short chain fatty acids separated chromatographically was determined by mounting an aliquot of the sodium salt of the fatty acid obtained after titration with sodium ethylate. An aliquot of the non-steam-distillable fraction containing the non-volatile fatty acids was mounted, and its C^{14} content determined.

Results

$C^{14}O_2$ Formation—The longer the chain length of the incubated fatty acid, the smaller were the $C^{14}O_2$ recoveries when a fatty acid was incubated as sole substrate (Table I). The effect of addition of glucose to the medium

varied with the chain length. The $C^{14}O_2$ recoveries from acetate were reduced more than 50 per cent, whereas those from butyrate and hexanoate were reduced about 40 and 20 per cent, respectively. In the case of octanoate-1-C^{14}, the addition of glucose to the medium slightly increased the $C^{14}O_2$ recoveries.

TABLE I

Incorporation of C^{14} of Acetate-1-C^{14}, Butyrate-1-C^{14}, Hexanoate-1-C^{14}, and Octanoate-1-C^{14} into CO_2 and Glycerides by Surviving Slices of Rat Mammary Gland, and Effect upon this Incorporation of Fortification of Medium with Glucose

500 mg. of mammary slices were incubated for 3 hours at 37.5°. The medium consisted of 4.5 ml. of bicarbonate buffer (free of calcium) and 0.5 ml. of the solution containing the sodium salt of the fatty acid. When glucose was added, the flasks contained 0.5 ml. of the fatty acid solution, 0.5 ml. of the glucose solution, and 4.0 ml. of the bicarbonate buffer. Gas phase, 95 per cent O_2 and 5 per cent CO_2. Duplicate flasks were incubated, and each figure recorded below is the average of two separate determinations.

Rat No.	Days postpartum	Labeled substrate	Unlabeled substrate	Per cent of added C^{14} recovered as	
		50 μmole	50 μmole	CO_2	Glyceride-C^{14}
1	7 (290)*	Acetate	None	7.0	1.0
		"	Glucose	3.0	17.9
		Butyrate	None	6.8	0.8
		"	Glucose	3.5	7.4
		Hexanoate	None	3.9	2.1
		"	Glucose	3.7	9.7
		Octanoate	None	1.0	1.5
		"	Glucose	1.8	39.0
2	16 (344)	Acetate	None	5.0	0.5
		"	Glucose	1.6	20.0
		Butyrate	None	3.7	0.3
		"	Glucose	1.9	6.6
		Hexanoate	None	2.0	1.5
		"	Glucose	1.5	15.4
		Octanoate	None	0.3	0.9
		"	Glucose	0.6	46.2
3	15 (344)	Acetate	None	3.1	0.3
		"	Glucose	1.5	17.6
		Butyrate	None	3.5	0.4
		"	Glucose	2.2	5.3
		Hexanoate	None	2.2	2.3
		"	Glucose	1.5	14.4
		Octanoate	None	0.7	1.0
		"	Glucose	1.0	33.0

* The weights of the rats, in gm., are given in parentheses.

Glyceride Formation—Little C^{14} was incorporated into glycerides when the short chain fatty acids served as *sole* substrate (Table II). The addition of glucose increased the incorporation of the C^{14} of all of the incubated fatty acids into glycerides. The highest glyceride-C^{14} values were observed with octanoate, the next highest with acetate, then with hexanoate, and the lowest with butyrate.

In one of the experiments, no C^{14} was found in phospholipides separated from the total lipide extracts (15).

Distribution of C^{14} among Glyceride Fatty Acids—Chromatographic separation of the glyceride fatty acids was performed only when glucose was added to the medium (Table II).

TABLE II

Distribution of Fatty Acid-C^{14} of Glycerides Obtained from Mammary Tissue Incubated with Acetate-1-C^{14}, Butyrate-1-C^{14}, Hexanoate-1-C^{14}, and Octanoate-1-C^{14}, Each Incubated with Unlabeled Glucose

For the experimental details, see the text.

Carboxyl-labeled fatty acid incubated with glucose	Per cent of C^{14} of hydrolyzed glyceride-C^{14} recovered as				
	Butyric acid	Hexanoic acid	Octanoic acid	Decanoic acid	Fatty acids containing more than 10 carbons
Acetate	0*	0	18.2	17.5	64
"	0*	2.3	8.1	21.8	67.6
Butyrate	0.1	0.8	15.5	32.0	51.5
"	0.5	4.0	22.3	25.5	47.4
Hexanoate	0*	44.6	10.6	8.0	35.9
"	0*	73.3	7.8	2.7	15.9
Octanoate	0*	0.5	98.4	0.5	0
"	0*	1.8	95.7	2.1	0.1

* Negligible.

Experiments with Acetate-1-C^{14} and Butyrate-1-C^{14}—Over 50 per cent of the isotope recovered in the glyceride fraction was located in fatty acids of chain lengths greater than 10 carbons. The 6- and 4-carbon fatty acids contained negligible amounts of C^{14} when acetate was the labeled substrate, and less than 1 per cent of the incubated C^{14} when butyrate was the labeled substrate.

Experiments with Hexanoate-1-C^{14}—Hexanoate-1-C^{14} was incorporated chiefly into the short chain fatty acids of the mammary gland glycerides, and most of the isotope was recovered in the 6-carbon fatty acid.

Experiments with Octanoate-1-C^{14}—Octanoate-1-C^{14} was incorporated solely into the short chain fraction of the isolated glycerides. About 40 times as much octanoate was incorporated into the mammary gland glyc-

erides in the presence of glucose as in its absence, and practically all of the C^{14} recovered in the glycerides was present in the 8-carbon fatty acids. The isolated octanoate-C^{14} obtained from the mammary gland glycerides contained all of the C^{14} in the carboxyl carbon. The decarboxylation of the octanoic acid was carried out as described by Anker (16).

DISCUSSION

The results of the present study demonstrate unequivocally that glucose promotes the *direct* esterification of octanoate by rat mammary gland. Thus, when octanoate-1-C^{14} was incubated with surviving mammary gland slices in a medium containing glucose, practically all of the C^{14} recovered in the glyceride fraction was present in the 8-carbon fatty acids, and degradation of those fatty acids revealed that all of the C^{14} was in the carboxyl carbon.

The data obtained with hexanoate show that glucose also stimulated the *direct* esterification of this fatty acid. Thus, 6-carbon fatty acids were found in large amounts as components of glycerides only when hexanoate was incubated in a glucose-containing medium. Since, in this experiment, the C^{14} of the hexanoate-1-C^{14} was also incorporated into glyceride fatty acids of chain lengths longer than 6 carbons, esterification may not be the exclusive action of the added glucose.

In the case of acetate and butyrate, *direct* esterification does not explain the stimulation of glyceride synthesis by glucose; practically all of the C^{14} recovered in the glyceride fraction was found in fatty acids containing 8 or more carbons.

In the experiments in which no glucose was added to the medium, it was observed that $C^{14}O_2$ production was highest from acetate and butyrate, lowest from octanoate, and intermediate from hexanoate. When glucose was added to the medium, the highest formation of *C^{14} long chain fatty acids* occurred in the experiments with acetate and butyrate, next with hexanoate, and least with octanoate. This correlation between $C^{14}O_2$ production in the absence of glucose and C^{14} long chain fatty acid formation in the presence of added glucose suggests that long chain fatty acids are formed in mammary gland principally by condensation of 2-carbon fragments rather than by elongation of previously existing short chain fatty acids. The $C^{14}O_2$ production can be considered an indication of the relative capacity of the mammary gland to degrade the fatty acids to acetyl S coenzyme A.

The question as to how glucose increased the glyceride-C^{14} formation from acetate and butyrate remains to be considered. Such an increase, as well as the stimulation of long chain glyceride formation from hexanoate

in the presence of glucose, may be explained either by a stimulation of the condensation of 2-carbon fragments or by an increase in the esterification of newly synthesized fatty acids. An increase in the latter process, as pointed out by Balmain and Folley (17), may serve to promote the further condensation of 2-carbon fragments by removal of the newly formed fatty acyl coenzyme A.

SUMMARY

1. Rat mammary gland slices were incubated, in the presence and absence of glucose, with the sodium salts of the following fatty acids: acetate-1-C^{14}, butyrate-1-C^{14}, hexanoate-1-C^{14}, and octanoate-1-C^{14}. The recoveries of the C^{14} in CO_2 and in the glyceride fraction were determined. The glyceride fraction was analyzed for C^{14}-labeled, short and long chain fatty acids.

2. In the case of octanoate, glucose promoted its *direct* esterification, and this appears to be the sole action of the carbohydrate in promoting lipogenesis from octanoate.

3. Glucose also promoted the direct esterification of hexanoate, but this is not the sole action of the carbohydrate in promoting lipogenesis from this short chain fatty acid.

4. *Direct* esterification did not account for the action of glucose on lipogenesis from acetate and butyrate. The C^{14} of acetate-1-C^{14} and butyrate-1-C^{14} was recovered in fatty acids containing 8 or more carbons. The possibility that glucose increased the esterification of newly synthesized fatty acids, thereby promoting further condensation of 2-carbon fragments, is considered.

5. The evidence presented here suggests that long chain fatty acid formation in rat mammary gland occurs primarily from condensation of 2-carbon units rather than from elongation of preexisting, short chain fatty acids.

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PROTEINS IN MULTIPLE MYELOMA

V. SYNTHESIS AND EXCRETION OF BENCE-JONES PROTEIN*

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Oral administration of amino acids labeled with N^{15} or with C^{13} to patients with multiple myeloma, followed by isolation of the abnormal serum and urinary proteins, has led previously to the conclusion that Bence-Jones protein is not derived by cleavage of circulating serum proteins nor by degradation of tissue proteins (1, 2). In point of fact, no evidence for any kind of precursor relationship between the two types of abnormal proteins was found. The data from these experiments also suggested that the Bence-Jones protein is formed rapidly *de novo* from free amino acids and is excreted rapidly. However, the stable isotopes proved unsuitable for the precise study of the rate of synthesis and excretion of Bence-Jones protein because they are too insensitive for intravenous administration at tracer levels and for measurement of the isotopic concentration of the unhydrolyzed protein. Thus, for the present work lysine, uniformly labeled with C^{14}, was chosen to facilitate the rate study. The lysine was injected into a subject who copiously excreted Bence-Jones protein but lacked an abnormal serum globulin. The urine was collected by urethral catheter, and the specific activity of the purified protein specimens was determined. The activity was maximal at 10 hours and thereafter declined rapidly. This indicates that the urinary protein was synthesized from the free amino acids rather than from preformed tissue proteins and that Bence-Jones protein, once released into the circulation, is treated like other nitrogenous excretory products, despite its size.

EXPERIMENTAL

Methods and Procedures

Serum and Urinary Proteins—The serum and urinary proteins were characterized by analysis in the Tiselius type electrophoresis apparatus and with the analytical ultracentrifuge (3, 4). The Bence-Jones protein was

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precipitated at 4° by dialysis against 3 M ammonium sulfate at pH 5.2. The heavy precipitate was removed by centrifuging and was washed twice with the salt solution. The precipitate was dissolved in water, and the solution was centrifuged till clear and then dialyzed against 0.9 per cent NaCl until free of sulfate ion. Aliquots were dialyzed against distilled water until free of chloride ion and then were lyophilized. A schlieren effect was noticed after several changes with distilled water in a preparation made prior to the isotopic study. Repeated changes and prolonged dialysis in the cold led to crystallization of the Bence-Jones protein as large sheets of crystals within the dialysis sac. However, the crystals dissolved on warming to room temperature. Although the crystallization was readily repeated, no attempt was made to crystallize the fourteen specimens used in the isotopic study.

Radioactive Measurements—L-Lysine labeled uniformly with C^{14} was obtained from the Nuclear Instrument and Chemical Corporation. Prior to administration the compound was dissolved in a 0.9 per cent NaCl-5.0 per cent glucose solution and was tested for toxicity and sterility. For determinations of radioactivity, the lyophilized protein was dissolved in 0.02 N NaOH, deposited on oxidized copper planchets, and dried to yield a uniform adhesive film. The planchets were counted with a gas flow counter (Atomic Instrument Company). Counts were made to a minimum of 1000 over background and were corrected for self-absorption. Respiratory CO_2 was also collected, and the specific activity and cumulative activity were kindly measured by Dr. George Okita by means of a vibrating reed electrometer. A later attempt to determine the specific activity of the urinary urea CO_2 by means of the urease reaction was unsuccessful because the samples available (from 2.5 hours on) had too little activity for accurate measurement.

In order to obtain an estimate of the excretion of the lysine that had been administered, the amino acids in 5 ml. urine samples were decarboxylated by reaction with ninhydrin at 100° in the presence of 15 mg. of glutamic acid as carrier. The CO_2 was trapped with $Ba(OH)_2$ and counted as $BaCO_3$ in an aluminum planchet. The data are presented for samples of infinite thickness for a planchet of 3.47 sq. cm. area. Since the lysine concentration of each sample of urine was not determined, correction for the carrier added could not be made.

Subject and Protocol

Subject[1]—The patient M. B. was admitted to the Argonne Cancer Research Hospital for study and terminal care. Relevant biochemical find-

[1] We are indebted to Dr. Robert Wissler and Dr. Benjamin Spargo of the Department of Pathology, University of Chicago, for the autopsy report. Details of the case history will be furnished on request.

ings were (1) anemia (hemoglobin of 7.4 gm. per cent), (2) hypoproteinemia (total serum protein of 4.8 gm. per cent with 1.7 gm. per cent globulins), and (3) renal insufficiency (urea clearance of 24, as calculated by the square root method, and elevated blood urea nitrogen and uric acid). The patient died of uremia 2 months after admission. Post mortem examination supported the diagnosis of multiple myeloma and revealed an extensive amyloidosis.

Protocol[2]—For the experiment, 300 μc. of L-lysine in 100 ml. of pyrogen-free solution were given intravenously over a 7 minute interval. There was no reaction. A complete urine collection by catheter was maintained for the next 13 hours. The first sample was taken at 30 minutes and the others at progressively increasing times. The catheter was removed, but complete urine collection at 4, 6, or 12 hour intervals was maintained for 90 hours with a total of thirteen specimens. A final specimen was taken on the 13th day. Because of the anemia and hypoproteinemia, the blood activity was not investigated. However, expiratory CO_2 samples were collected by Dr. George Okita over a period of 24 hours. An aliquot of each urine sample was frozen for later study, and the remainder was used immediately for preparation of the Bence-Jones protein.

Owing to the patient's anorexia, a nitrogen balance study was unsuccessful. During the 4 days of the experiment, the daily protein intake rose from 40 to 99 gm. A week later it had fallen to 10 gm. However, the daily excretion of Bence-Jones protein was almost constant (about 10 gm.).

Results

Serum and Urinary Proteins—The electrophoretic pattern of the patient's serum in Veronal buffer, pH 8.6 (Fig. 1, *A*), reveals the absence of a predominant globulin component of the type observed in about two-thirds of the cases of multiple myeloma (3). Area analysis indicated that percentagewise the serum protein distribution was as follows: albumin 53.8, α_1-globulin 5.3, α_2-globulin 17.0, β-globulin 19.4, and γ-globulin 4.6. Thus, α_2- and β-globulin were significantly elevated percentagewise (α_2 by 2-fold), whereas γ-globulin had diminished to one-third of normal on this basis and to one-fifth on an absolute basis.

Since the Bence-Jones protein had a mobility close to that of β-globulin (compare Fig. 1, *A* and *B*), the relative increase in this peak may result from the presence of Bence-Jones protein (also from a slight hemolysis).

The Bence-Jones protein appeared to be quite homogeneous by the criteria applied. It moved with a single sharp boundary both in electrophoresis and ultracentrifugation (Fig. 1, *B* and *C*) and crystallized readily (Fig. 2). Amino end-group analysis by the dinitrophenyl (DNP) method revealed

[2] Dr. Milton Weiner aided in devising the protocol and in the medical management of the patient, under the direction of Dr. Robert Hasterlik and Dr. George V. LeRoy.

only trace amounts of ether-soluble DNP-amino acids, a total of about 0.1 mole per mole of protein. Thus, this protein is one of a group of crystallizable Bence-Jones proteins that are devoid of end-groups detectable by the DNP method (5). Further work on this problem is in progress. In the Veronal buffer, pH 8.6, the Bence-Jones protein had a mobility of -2.6×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$ and a sedimentation constant of 3.4 Svedberg units, values similar to those reported for proteins D and G (4). It was in the same antigenic group as protein G.[3]

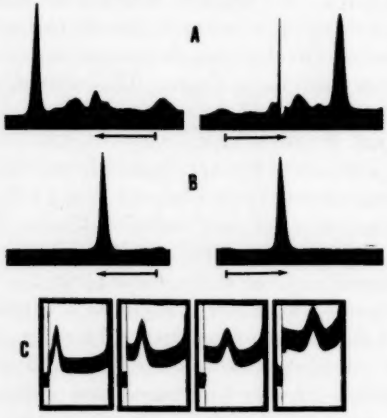

FIG. 1. Electrophoretic diagrams of the serum proteins (*A*) of subject M. B. and of a specimen of the radioactive Bence-Jones protein (*B*) and ultracentrifugal diagrams of the latter protein (*C*). All experiments were performed in Veronal buffer, pH 8.6, ionic strength 0.1. The arrows in *A* and *B* indicate the origin and direction of migration. Since the photographs of *A* and *B* were taken after 200 minutes at the same current (16 ma.), the mobilities can be compared by visual inspection. In *C*, sedimentation was from right to left, with photographs taken at 32 minute intervals at a rotor speed of 59,780 r.p.m.

Expiratory CO_2—According to current views (6), the first steps in the metabolism of the carbon chain of lysine involve deamination, followed by oxidative decarboxylation yielding CO_2. The latter would rapidly appear in the expiratory CO_2. These reactions produce glutaric acid, the further metabolism of which is unknown. From Fig. 3, it can be seen that there is a rather rapid initial expiration of $C^{14}O_2$ with a maximum at about 2.5 hours. Although the decline in activity for the first 8 hours is almost exponential (half time of about 6 hours), this is followed by a slower nonlogarithmic decrease in specific activity. The cumulative activity of ex-

[3] Antigenic group A, according to Dr. Leonhard Korngold of the Sloan-Kettering Institute for Cancer Research (private communication).

piratory $C^{14}O_2$ was still rising at 24 hours, by which time 3.9 per cent of the injected activity had been exhaled.

In the choice of C^{14}-amino acids for studies of protein synthesis, one must consider whether to use specifically or randomly labeled compounds. The choice of an essential *versus* a non-essential amino acid is also important, for Penn, Mandeles, and Anker (7) have demonstrated that the apparent turnover time varies with the nature of the amino acid selected as tracer. Hence, in an experiment with a different patient, DL-glutamic acid-1-C^{14} was chosen to investigate the possible precursor relationships of the abnormal serum globulin and of the Bence-Jones protein. Although the fractionation of the serum proteins is not yet complete, data on the specific

FIG. 2. Photomicrographs of crystals of Bence-Jones protein from patient M. B. (53 ×).

activity of the expiratory CO_2 and the Bence-Jones protein are available for comparison with results obtained after the administration of lysine.

Glutamic acid-1-C^{14} would be deaminated readily to yield α-ketoglutarate, which in turn would be decarboxylated rapidly via the citric acid cycle, yielding $C^{14}O_2$ and unlabeled succinic acid (6). In accord with expectation, the specific activity of the expiratory $C^{14}O_2$ was maximal within the first half-hour. Not only did this maximum occur 2 hours earlier than with the uniformly labeled lysine, but it was also almost four times greater, although the two subjects were given about equivalent activity in the L form (compare Fig. 4). (On the other hand, the maximal specific activity of the Bence-Jones protein of the subject given lysine was seven times greater than that of the patient given glutamic acid.)

Since the specific activity-time curves for expiratory $C^{14}O_2$ reflect the blood level of the tracer amino acid, they are a useful index in the absence

of kinetic data on the latter quantity. It is evident that the blood level of radioactivity is maintained longer in the case of lysine, leading to a more efficient incorporation in the protein, but that glutamic acid-1-C^{14} would give a more instantaneous picture of protein synthesis.

Bence-Jones Protein—The specific activity curve for the Bence-Jones protein (Fig. 5) demonstrates a rapid uptake and decline of isotopic amino acid in the excreted protein. The first specimen of Bence-Jones protein was devoid of radioactivity (obtained at 30 minutes after injection, but

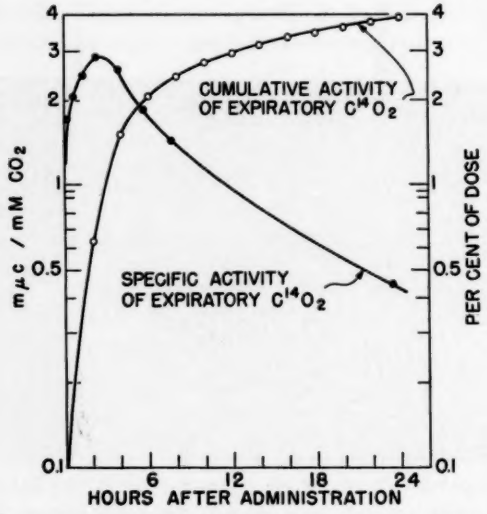

FIG. 3. Semilogarithmic plot of the specific activity (left ordinate) and cumulative activity (right ordinate) of the expiratory $C^{14}O_2$ after injection of 300 μc. of L-lysine-C^{14} (measurements by Dr. George Okita of the Argonne Cancer Research Hospital).

omitted from the graph of Fig. 5). After this lag, the activity rose sharply to a maximum at 9.5 hours. Thereafter, it fell off steeply for a day, and then more slowly. The declining curve was not truly exponential but appeared to have two logarithmic segments. The first, terminating at 36 hours, corresponded to a half time of decline of 15 hours; the second, ending at 90 hours, was equivalent to a half time of about 60 hours. A specimen taken 8 days later had unexpectedly high activity (79 c.p.m. per mg. of protein).

The data of Fig. 5 are alone inadequate to give a complete picture of either the rate or the cumulative balance of the excretion of the labeled Bence-Jones protein. The rate is clearly a function of the urine volume

and protein concentration as well as of the specific activity. The balance is incomplete because urine collection was stopped on the 4th day. How-

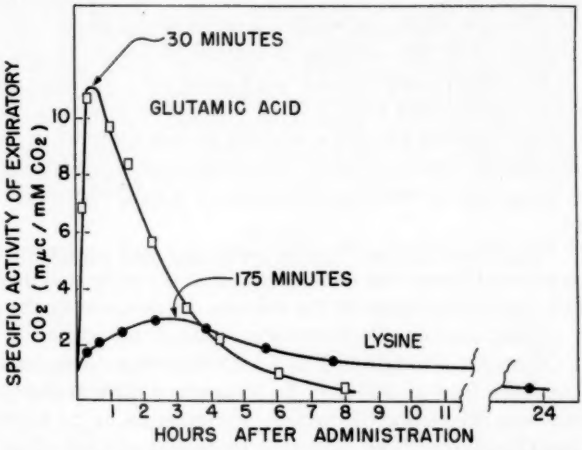

FIG. 4. Comparison of the specific activity-time curves for the expiratory CO_2 in two subjects, the one (M. B.) given 300 μc. of L-lysine-C^{14} and the other injected with about 450 μc. of DL-glutamic acid-1-C^{14} (measurements by Dr. George Okita of the Argonne Cancer Research Hospital).

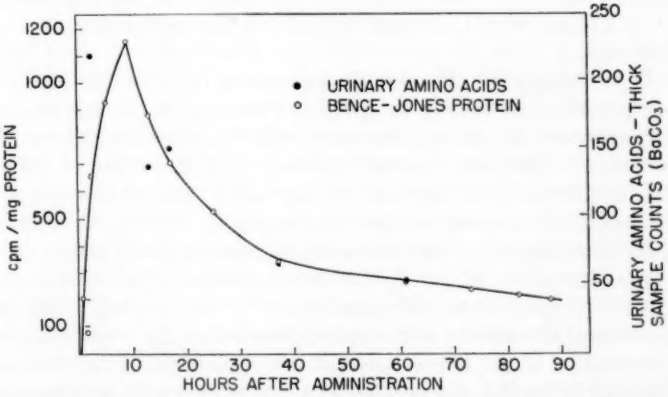
URINARY AMINO ACIDS
BENCE-JONES PROTEIN
cpm / mg PROTEIN
URINARY AMINO ACIDS – THICK SAMPLE COUNTS (BaCO3)
HOURS AFTER ADMINISTRATION

FIG. 5. Specific activity-time curves of the Bence-Jones protein isolated from the urine of subject M. B. and of the $BaCO_3$ obtained by reaction of the urine with ninhydrin. The initial sample of urine, collected by catheter for the first 30 minutes after injection, proved to be inactive and is not represented on the curve.

ever, the hourly rate could be calculated for the first 90 hours from the total protein recovered in each specimen and from its activity. A histo-

gram of the activity (total counts) excreted per hour was constructed. The general shape of the curve was similar to that of Fig. 5, with a maximum at the 10th hour. Although there was more fluctuation in the values at the later intervals, no diurnal variation could be discerned. Of the total activity eliminated in the urine via protein in 90 hours, 19 per cent was excreted in the 4 hour period from 9 to 13 hours after injection and 45 per cent in the 12 hour period from 5 to 17 hours after injection. It can be estimated that the latter amount is as great as that excreted in the 12 day period following the time covered by Fig. 5 (estimated from the logarithmic plot of declining activity and the assumption of a daily excretion of 10 gm. of protein).

Urinary Excretion of Lysine-C^{14}—The possibility that an appreciable fraction of the injected lysine was excreted *per se* in the urine was investigated by ninhydrin decarboxylation of the urinary amino acids in the presence of carrier. Unfortunately, the frozen specimens of the early urine samples were lost. However, the data in Fig. 5 are adequate to reveal the level of excretion and the rate of decline. In comparison with D-glutamic acid, little L-lysine was excreted in the urine.[4] For example, at 2.5 hours the activity of $BaCO_3$ obtained from ninhydrin treatment of 5 ml. of urine, with 15 mg. of glutamic acid added as carrier, was 220 c.p.m. for subject M. B., whereas, at the same time and with only 0.3 ml. of urine, and the same weight of carrier, the activity was 12,800 c.p.m. in the subject given DL-glutamic acid-1-C^{14}. Thus, the amino acid activity of the two urines differed by a factor of 870, although the protein was more radioactive in the present case.

Tests for Adsorption—Because the adsorption of lysine by proteins has been represented as leading to spurious results in tracer experiments of protein synthesis (8), several tests were made to assure the absence of this phenomenon. The tests involved (*a*) dialysis of a mixture of unlabeled protein and lysine-C^{14} of high activity against a series of changes of 0.15 M NaCl until the external solution had negligible activity, (*b*) dialysis of labeled protein against a solution containing inactive lysine in 0.15 M NaCl, and (*c*) a comparison of the $BaCO_3$ activity following the action of ninhydrin on the hydrolyzed and unhydrolyzed labeled protein. The period of dialysis and the number of changes approximated the conditions used in the preparation of the Bence-Jones protein specimens. From the results summarized in Table I, Experiment I, it can be seen that dialysis, even in the absence of lysine outside the sac, effectively reduces the radioactivity inside the sac to a negligible value. Similarly, in Experiment II, there

[4] Virtually all the urinary amino acid activity of the patient given DL-glutamic acid was in the form of the unnatural isomer; *e.g.*, in a urine sample yielding $BaCO_3$ with an activity of 6520 c.p.m. by ninhydrin decarboxylation, the activity was only 5 c.p.m. on enzymatic decarboxylation.

was no significant change in the specific activity of the labeled protein upon dialysis against free, inactive lysine. In addition, no activity above background was found in the $BaCO_3$ obtained from the reaction of ninhydrin in a solution containing labeled protein and inactive carrier amino acid, whereas the specific activity of the $BaCO_3$ obtained from ninhydrin decarboxylation of the same specimen of protein (and carrier) after hydrolysis was 696 c.p.m. at infinite sample thickness. We conclude that the Bence-Jones protein was labeled only by true incorporation of the isotopic lysine through peptide bonds.[5]

TABLE I

Test for Adsorption of Lysine-C^{14} by Bence-Jones Protein

Experiment No.	Dialysate	No. of changes	C.p.m. per mg. protein
I. Unlabeled protein and lysine-C^{14}	0.15 M NaCl	10	11
Change dialysis sac	0.15 " "	4	6.6
Control	No dialysis		21,500
II. Labeled protein	10 mg.% lysine in 0.15 M NaCl	3	
	0.15 M NaCl	2	518
Control	No dialysis		525

DISCUSSION

Bence-Jones proteinuria is a characteristic but not an invariable finding in multiple myeloma. Whereas some patients have no detectable Bence-Jones protein in the urine, others excrete up to one-half their daily nitrogen intake via this abnormal metabolic product. Two other aberrations in protein metabolism may occur in this disease, (1) a hyperglobulinemia resulting from the formation of individually characteristic, abnormal serum globulins (3, 9–12) and (2) the deposition of protein in the tissues (amyloidosis). Like the myeloma globulins, the Bence-Jones proteins are not identical; indeed, they appear to differ with each individual (4, 5). Although the response of a given patient is characteristic and consistent, no correlation of the protein abnormalities can yet be made with clinical symptoms, diagnostic findings, plasma cell type, or longevity.[6]

The variety of abnormal plasma and urinary proteins formed by different

[5] A similar conclusion was reached after more extensive tests in the case in which a patient was given DL-glutamic acid-1-C^{14}. In this instance, in which one-half the activity is excreted via the D isomer, the dialysis tests and the lack of reaction with ninhydrin were confirmed by the failure of glutamic acid decarboxylase to reduce the activity of the excreted protein.

[6] Kubota, C., Schwartz, S. O., and Putnam, F. W., from data obtained in a survey of 78 cases (in press; see also (13)).

patients with multiple myeloma (3, 4, 9–11) requires that investigation of protein synthesis in this disease be undertaken in several patients with representative aberrations. In preceding experiments (1, 2), two patients were studied with use of glycine labeled with N^{15} or C^{13}. Both subjects had hyperglobulinemia, the one owing to a cryoglobulin of the γ type, the other from an abnormal β-globulin. Each excreted a single Bence-Jones protein, distinguishable from the other. Despite these individually characteristic abnormalities in protein synthesis, the same conclusions were drawn for the two subjects; namely (1) there was no apparent metabolic relationship between the abnormal protein of the plasma and that of the urine, and (2) in each case the Bence-Jones protein was synthesized rapidly and excreted rapidly and appeared to be formed *de novo* from free amino acids rather than by degradation of serum or tissue proteins.

The present investigation extends these observations to a subject of a third type, *i.e.* one who copiously excretes a highly homogeneous Bence-Jones protein but has hypoglobulinemia. The previous experiments, based on 24 hour urine collections, demonstrated that oral administration of a large dose of C^{13}- or N^{15}-labeled amino acid (5 to 20 gm. during an 8 hour period) was followed within 24 to 48 hours by a maximum in the C^{13} or N^{15} content of the urinary protein. In the present case, it is shown that intravenous infusion of a tracer dose of lysine-C^{14} (about 50 mg.) leads to a maximum in the specific activity of the excreted protein within 10 hours. Upon comparison of the activity curves for expiratory CO_2 in the case of lysine and glutamic acid (Fig. 4), one might anticipate a sharper decline in the activity of Bence-Jones protein (and probably an earlier maximum) if the more rapidly metabolized amino acid had been used as the tracer.

Although this experiment gives a more complete picture of the rate of formation and excretion of Bence-Jones protein, the kinetics of these processes cannot be calculated accurately from the data presented. The rate of change in activity of the Bence-Jones protein is a function of at least four factors: (1) the rate of decline in the specific activity of the free lysine, (2) the rate of synthesis of the protein, (3) the rate of its release into the circulation, and (4) the rate of renal excretion. The time required for an amino acid residue to pass from the free amino acid pool to the circulating serum protein has been defined as the "transit time." The transit time is a function of the first three factors listed, and in rabbits, turtles, and the horseshoe crab it is approximately 30 minutes at 37° (8). As judged by the first appearance of radioactive protein in the urine, the maximal time lag for synthesis *and excretion* of Bence-Jones protein is 30 to 60 minutes. This short time lapse was quite unforeseen when the experiments with stable isotopes were planned (1, 2). Although the time lags for the synthesis of serum proteins and the synthesis and elimination of Bence-Jones

protein are comparable, the curves for the decline in activity are not. The serum proteins "turn over" among the most rapidly of all tissue proteins, and, in man, serum proteins have a turnover time of from 5 to 20 days (8, 13). However, it is evident from the even more rapid activity decline illustrated in Fig. 5 that Bence-Jones protein is not derived from serum or from other preformed tissue proteins.

With regard to the first factor above, the specific activity of free lysine is reflected both by the activity of the urinary amino acid (Fig. 5) and by the activity of expiratory CO_2 (Figs. 3 and 4). Neither of these activities declines exponentially, nor does the activity of the Bence-Jones protein (Fig. 5). The similarity in the declining portions of the three curves is evidence, then, that the Bence-Jones protein is formed *de novo* from the administered amino acid. The data of Fig. 5 also suggest that the Bence-Jones protein is not withheld appreciably more by the kidney than is lysine (or its ninhydrin-reacting metabolites). This finding, though surprising, is compatible with the previous report that N^{15} is excreted as rapidly by way of incorporation into Bence-Jones protein as via metabolism to urea (2). Judging by comparison of the rising portions of the activity curves of Figs. 3 and 5 and their respective maxima, one concludes that it takes about 6 hours longer for lysine to be incorporated into Bence-Jones protein and for the latter to be excreted than it does for lysine to be metabolized to CO_2 and the latter exhaled.

Half of the activity excreted by way of labeled protein within the first 90 hours and about one-quarter of that so excreted within the first 2 weeks were voided in the 12 hour interval beginning 5 hours after the injection. Now, since labeled protein must have been made continuously, though with rapidly decreasing activity, it is clear that the bulk of the Bence-Jones protein made and released into the circulation within any given interval is excreted in the ensuing 12 hours. In other words, circulating Bence-Jones protein does not participate actively in the metabolic pool of nitrogen, but is treated like low molecular weight nitrogenous excretory products. The sharp maximum and the rapid decline in the curve of Fig. 5 preclude the presence of a large pool of Bence-Jones protein that undergoes turnover analogous to serum proteins.[7] The question whether some of the Bence-Jones protein is deposited in an inert form in amyloid tissue may be resolved when analyses of tumor masses and amyloid deposits are completed.

Since skin tests with the purified protein yielded no untoward reaction in the subject, it had been planned to measure the renal clearance and rate of excretion by reinjection of the labeled protein. However, the patient's

[7] Under similar conditions serum proteins in man exhibit a similar lag in activity, a slower rise, a much broader maximum, and a considerably slower decline (unpublished observations, F. W. Putnam, F. Meyer, and A. Miyake).

condition did not warrant the experiment. Such an experiment has taken place with the patient given glutamic acid-1-C^{14}, and it was also possible to measure the activity of the L-glutamic acid of serum and of the γ-globulin. The data, which are almost complete, are fully compatible with the conclusions drawn above.

We are greatly indebted to Dr. Robert Hasterlik, Dr. George V. LeRoy, and Dr. Leon Jacobson of the Argonne Cancer Research Hospital for their great interest and unceasing cooperation in this experiment.

SUMMARY

The rate of synthesis and excretion of Bence-Jones protein has been studied by intravenous injection of lysine uniformly labeled with C^{14}, followed by measurement of the specific activity of the protein isolated from successive urine samples. Urinary excretion of the labeled amino acid was estimated from the CO_2 obtained by the ninhydrin reaction. The specific activity of expiratory CO_2 was also determined and compared with the activity of expiratory CO_2 after injection of DL-glutamic acid-1-C^{14} in another subject. After a 30 minute lag period, the radioactivity of the urinary protein rose sharply to a maximum at 10 hours and then fell off abruptly in a non-logarithmic fashion. The activity of expiratory CO_2 was maximal at 2.5 hours and also declined rapidly and non-exponentially. The data confirm the earlier conclusion that Bence-Jones protein is rapidly synthesized from the free amino acids rather than from tissue protein precursors. Bence-Jones protein formed in any given interval is excreted for the most part within the ensuing 12 hours.

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STUDIES ON THE SULFUR METABOLISM OF TISSUES CULTIVATED IN VITRO

II. OPTICAL SPECIFICITIES AND INTERRELATIONSHIPS BETWEEN CYSTINE AND METHIONINE

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Previous studies from this Laboratory (1) have shown that chick embryonic heart fibroblasts cultivated *in vitro* in a completely synthetic medium (2, 3) have a critical requirement for L-cystine which cannot be satisfied by methionine. For optimal survival of these tissues, however, the presence of both cystine and methionine was found necessary. In view of the importance of the sulfur amino acids for the maintenance of cell survival, it seemed logical to extend these studies to a consideration of the quantitative interrelationships between cystine and methionine and to determine the optical specificities involved. The results reported in the present communication emphasize the complexity of the cystine-methionine requirement and show that D-methionine possesses activity equal to that of the L isomer, but that D-cystine is completely inactive.

Materials and Methods

Tissue cultures for this study were prepared from the heart muscle of 11 day-old chick embryos. The hearts were chopped to a fine pulp with scissors, moistened with synthetic medium, and small portions were transferred to 18 × 150 mm. Pyrex test-tubes by a capillary pipette. Each culture contained from 1 to 2 mg. of tissue, wet weight, as determined by frequent weighings of replicate samples and by total protein measurements (4). After 25 to 30 minutes, the tissue fragments became fixed to the glass, without the use of plasma, the synthetic medium was added, and the cultures incubated at 37° by the conventional roller tube method (5). Full details of the culture procedures have been reported previously (6, 7).

The basic synthetic medium used throughout these experiments was M 150 (2, 3), which supports the survival of freshly explanted chick embryonic heart fibroblasts for approximately 35 days. Media deficient in sulfur-containing amino acids were prepared by omitting from the formula L-cystine, DL-methionine, L-cysteine, and glutathione. Various proportions of L- or D-cystine and L- or D-methionine were then added to the deficient media. The concentration range employed was 0.01 to 100.0 mg. per liter.

This range had been found previously (1) to be the most suitable for studies with the sulfur amino acids. Since the previous studies had shown that inorganic sulfate had no effect on the survival time of the chick embryonic heart cultures, changes in the synthetic media were restricted to organic sulfur compounds, and the basic inorganic salt solution (3) was not altered. All the compounds used in the media were of the highest grade obtainable commercially and were employed without further purification. Paper chromatographic analyses (8) showed that the individual amino acids used were relatively uncontaminated with other ninhydrin-positive materials. All media were prepared at frequent intervals and sterilized by passage through ultrafine fritted glass filters.

The experimental design and the method of evaluating the cultures have been described in detail in previous publications (1–3, 7). All the figures reported represent average survival times, calculated from two or more separate experiments, involving at least twenty cultures at each test level. The significance of differences in survival times was calculated by the standard t test, performed through the courtesy of Mr. E. J. Hamilton of this Laboratory.

Results

Relationship between L-*Cystine Response and* L-*Methionine Concentration*—Synthetic medium M 150 was prepared from which the cystine, methionine, cysteine, and glutathione had been omitted. From this sulfur-deficient medium, basic solutions containing selected concentrations of L-methionine were prepared. Graded levels of L-cystine were then added to each of these basic solutions and the cystine response curves determined (Fig. 1).

In the absence of methionine (Curve A), a moderate cystine response is apparent. In the presence of 0.01 mg. per liter of L-methionine (Curve C), a marked cystine response is obtained in which the survival times of the cultures correspond with those normally obtained in the complete medium M 150. Increased amounts of L-methionine (0.1, 1.0, 10.0, and 100.0 mg. per liter) yielded response curves indistinguishable from that obtained at the 0.01 mg. per liter concentration. The decline in the survival time (Curve B) shown when 100.0 mg. per liter of L-cystine was added to the media containing 100.0 mg. per liter of L-methionine was not statistically significant. Since there was no graded effect due to the different methionine levels tested, further extension of the concentration range did not appear necessary.

Relationship of Optical Configuration to Methionine Activity—The marked effect of L-methionine in supplementing the cystine response in the chick embryonic heart cultures made it of interest to investigate the possible activity of the D isomer. Sulfur-deficient synthetic media were prepared

as before and divided into two portions. To one portion were added 10.0 mg. per liter of L-cystine, while the second portion was kept without cystine. In these two basal media, graded levels of either L- or D-methionine were incorporated over the concentration range of 0.01 to 100.0 mg. per liter. The survival times of the cultures in these various media are presented in Fig. 2.

Curves A and B show that, in the absence of cystine, neither L- nor D-methionine has any significant effect on the survival of the chick embry-

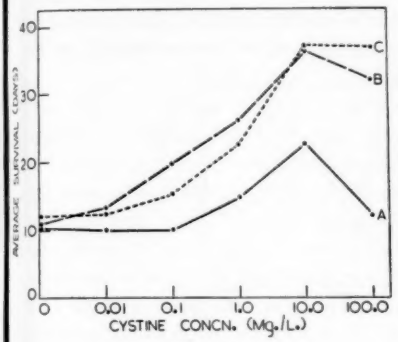

FIG. 1

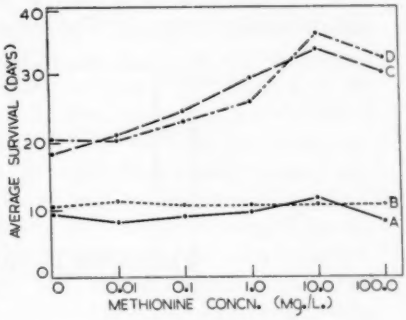

FIG. 2

FIG. 1. Response of tissues cultivated in sulfur-deficient media to graded concentrations of L-cystine. Curve A, L-cystine response in the absence of methionine; Curve B, L-cystine response in the presence of 100.0 mg. per liter of L-methionine; Curve C, L-cystine response in the presence of 0.01 mg. per liter of L-methionine.

FIG. 2. Response of tissues cultivated in sulfur-deficient media to graded concentrations of methionine. Curve A, L-methionine response in the absence of L-cystine; Curve B, D-methionine response in the absence of L-cystine; Curve C, L-methionine response in presence of 10.0 mg. per liter of L-cystine; Curve D, D-methionine response in the presence of 10.0 mg. per liter of L-cystine.

onic heart cultures. In the presence of adequate cystine, both L-methionine (Curve C) and D-methionine (Curve D) effectively supplement the cystine response. In both cases, the survival time of the cultures is equal to that found in the complete M 150. Since the response curves for D-methionine are indistinguishable from those of the L isomer, it would appear that the activity of the two isomers is identical in this biological system.

Relationship of Optical Configuration to Cystine Activity—In view of the activity found with D-methionine, the optical specificity of the cystine requirement was investigated. Sulfur-deficient media were prepared as before and divided into two portions. To one portion were added 30.0 mg. per liter of DL-methionine, the level normally present in the complete me-

dium M 150. The second portion was kept without methionine. Into these two basal media, graded levels of either L- or D-cystine were incorporated over the concentration range of 0.01 to 100.0 mg. per liter. In additional experiments, sulfur-deficient media were prepared and divided into two basal solutions, to one of which were added 10.0 mg. per liter of L-methionine and to the other 10.0 mg. per liter of D-methionine. Graded concentrations of L-cystine over the usual range were then incorporated in these two basal media and the survival times of the chick embryonic heart cultures determined. The results of these experiments are presented in Fig. 3.

In the absence of methionine, D-cystine had no effect on the survival of the cultures (Curve A). The same negative result was obtained when

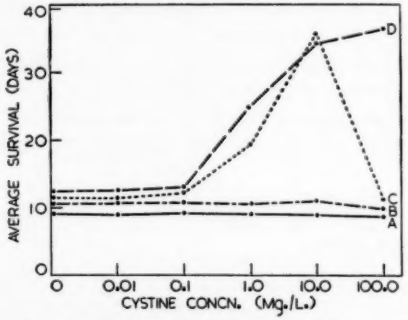

FIG. 3. Response of tissues cultivated in sulfur-deficient media to graded concentrations of cystine. Curve A, D-cystine response in the absence of methionine; Curve B, D-cystine response in the presence of 30.0 mg. per liter of DL-methionine; Curve C, L-cystine response in the presence of 10.0 mg. per liter of D-methionine; Curve D, L-cystine response in the presence of 10.0 mg. per liter of L-methionine.

D-cystine levels were tested in the presence of methionine (Curve B). It is apparent that D-cystine is completely inactive in some metabolic process essential for the survival of chick embryonic heart cultures. Since L-cystine exerts a marked effect on culture survival (Fig. 1), the optical specificity of the tissue culture requirement for the L isomer is clearly established.

In contrast to the negative findings with D-cystine, the addition of graded levels of L-cystine to synthetic media containing 10.0 mg. per liter of L-methionine produces the characteristic cystine response (Curve D), as reported previously (1). When the basal synthetic medium contains D- rather than L-methionine, one major difference in the cystine response becomes apparent (Curve C). Maximal survival of the tissue cultures is obtained at 10.0 mg. per liter of L-cystine, as observed previously, but the survival is decreased very sharply at 100.0 mg. per liter. It is evident that D-methionine is as effective as L-methionine in eliciting the maximal cystine

response, but that the combination of high concentrations of both L-cystine and D-methionine completely nullifies the beneficial effect.

DISCUSSION

The results of the present experiments, which are based on approximately 3000 cultures, emphasize the complex quantitative relationships that exist between cystine and methionine in the nutritional requirements of chick embryonic heart tissues cultivated *in vitro*. Moderate culture survival occurs in the absence of methionine, provided that adequate cystine is present in the medium. For maximal culture survival in the synthetic medium, methionine is necessary in addition to cystine. The amount of methionine required in the presence of adequate cystine may be as low as 0.01 mg. per liter, but the complete omission of methionine reduces the culture survival period by approximately 40 per cent. In the presence of L-methionine, optimal culture survival is obtained by the addition of 10.0 to 100.0 mg. per liter of L-cystine. If the medium contains D-methionine, optimal culture survival is obtained by the addition of 10.0 mg. per liter of L-cystine, but increasing the cystine concentration to 100.0 mg. per liter nullifies the beneficial effect. In the latter case, the survival of the cultures is equivalent to that obtained in media completely devoid of sulfur-containing amino acids. It would appear that, in the preparation of synthetic media for tissue cultures, the balance between cystine and methionine is a critical factor, especially if racemic compounds are used. Both cystine and methionine have recently been classed as essential for the L strain (9) and the HeLa strain (10), but no interrelationships between these two amino acids were reported. It is possible that the presence of serum in the experimental media employed may have obscured the delicacy of the amino acid responses. The present experiments, in which completely synthetic media were used, show that it is possible to obtain quantitative response curves in tissue cultures and to determine the interrelationships of individual amino acids.

The major differences in survival times of the chick embryonic heart cultures observed with various proportions of cystine and methionine in the synthetic medium make it possible to relate biological activity to the optical configuration of these compounds. L-Cystine prolonged the survival time of the cultures whether or not methionine was present in the medium, but the D isomer was completely ineffective under both conditions. In the presence of an adequate concentration of L-cystine, L- and D-methionine produced identical response curves, indicating comparable activity. In the absence of cystine, both isomers of methionine were ineffective. These experiments clearly establish that the tissue cultures have a specific requirement for L-cystine, but that the requirement for methionine is a

supplementary one which can be satisfied equally by the L or D isomer. This finding is in good agreement with the earlier observation by Grau and Almquist (11) that DL-methionine was equivalent to L-methionine for growth of the chick. Recent studies on the amino acid requirements of man (12) have shown that D-methionine, in contrast to the behavior in man of the D isomers of all other essential amino acids, was as effective as DL-methionine in the maintenance of nitrogen equilibrium. It should be pointed out that the present studies appear to be the first in which the pure L isomer has been compared directly with the D isomer. They therefore provide a quantitative evaluation of the unusual activity of D-methionine in biological systems.

The observation that chick embryonic heart cultures have a specific requirement for L-cystine but a non-specific one for methionine suggests that only part of the methionine molecule may be functionally active in this biological system. The basal synthetic medium employed throughout these studies, M 150, contains a relatively high concentration of choline (0.5 mg. per liter). Recent experiments have shown (unpublished data) that the cystine and methionine relationship is altered by the omission of choline from this medium. This suggests that the supplementary requirement for methionine may possibly be related to the supply of labile methyl groups.

The reference synthetic medium, M 150, contains 20.0 mg. per liter of L-cystine and 30.0 mg. per liter of DL-methionine. Both the cystine and methionine concentrations are, therefore, close to the optimal effective levels established in the present studies. The relative amino acid concentrations in this medium were originally calculated from published tables of analyses of a variety of animal proteins (13). It is interesting to note that the quantitative interrelationships between cystine and methionine established in the present experiments approximate the balance between these two amino acids found in most animal proteins. It is hoped that intensive studies of metabolic pathways, under precisely controlled conditions in tissue cultures, may help to explain the quantitative aspects of the balance found *in vivo* by showing the effects of imbalance on individual tissues.

SUMMARY

1. Freshly explanted chick embryonic heart tissues cultivated *in vitro* in a synthetic medium devoid of sulfur amino acids showed a graded response on the addition of cystine but not methionine. For maximal survival of the cultures, supplementation of the cystine with methionine was necessary. In the presence of adequate cystine (10.0 to 100.0 mg. per liter), 0.01 mg. per liter of L-methionine produced optimal culture survival.

2. The tissue cultures were found to possess a specific requirement for L-cystine, but D-cystine was completely inactive.

3. In the absence of cystine, neither L- nor D-methionine had any effect on the survival of the cultures. In the presence of 10.0 mg. per liter of L-cystine, L- and D-methionine showed equivalent supplementary activity.

4. The combination of a high concentration of L-cystine (100.0 mg. per liter) with the same concentration of D-methionine reduced the survival of the cultures to that obtained in the complete absence of sulfur amino acids.

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DIALKYLFLUOROPHOSPHATASES OF MICROORGANISMS*

II. SUBSTRATE SPECIFICITY STUDIES

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Enzymes which hydrolyze diisopropyl fluorophosphate (DFP) and related compounds are widely distributed in animal tissues and microorganisms (1–4). These dialkylfluorophosphatases (DFPases) have been shown to have different characteristics as determined by their substrate specificities and the effects of metal ions (5–11).

Previous studies with DFPases of microorganisms (4) were limited to DFP as the only substrate. The present investigation was carried on to characterize the DFPases in microorganisms by using a number of organophosphorus compounds as substrates.

Materials and Methods

Escherichia coli, *Pseudomonas fluorescens*, *Streptococcus faecalis*, and *Propionibacterium pentosaceum* were used in these studies. In previous papers, the procedures for growing and storing the organisms and the method of determining the rates of hydrolysis of organophosphorus compounds were presented (4, 8).

The following substances were tested as DFPase substrates: diisopropyl fluorophosphate,[1] diethyl and dibutyl fluorophosphates,[2] tetraethyl pyrophosphate,[3] tetra-*n*-propyl pyrophosphate,[4] tetraisopropyl pyrophosphate,[4] bis (dimethylamino) fluorophosphate,[5] tetrakis (dimethylamino) pyrophosphate,[6] triethyl phosphate,[6] *O*,*O*-dimethyl-2,2,2-trichloro-1-hydroxyethyl phosphate,[7] *O*,*O*-dimethyl-*O*-3-chloro-4-nitrophenyl thiophosphate, *O*,*O*-diethyl-*O*-4-nitrophenyl phosphate,[8] and *O*,*O*-diethyl-*p*-nitrophenyl thio-

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[1] Provided by the United States Army, Medical Division, Chemical Corps.

[2] Prepared from chlorophosphates donated by the Victor Chemical Works, Chicago, Illinois.

[3] Gift of the Niagara Chemical Company.

[4] Gift of the Victor Chemical Works.

[5] Donated by Dr. R. L. Metcalf.

[6] Donated by the Virginia-Carolina Chemical Company.

[7] Donated by Chemagro Corporation, New York.

[8] Donated by Dr. S. A. Hall, United States Department of Agriculture.

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phosphate.[6] Substrate concentrations were 25 mM for experiments with mixed substrates and 10 mM in all other cases. When inhibitors were used,

TABLE I

Hydrolyses of Organophosphorus Compounds by Microorganisms

The rate of hydrolysis = microliters of CO_2 per 100 mg. of organism per hour. Substrate concentrations, 10^{-2} M. All the determinations were made in the presence of 10^{-4} M Mn^{++}. The figures in parentheses represent the percentage rate of DFP hydrolysis.

Compound	Rate with organism			
	E. coli	*P. fluorescens*	*P. pentosaceum*	*S. faecalis*
Diisopropyl fluorophosphate (DFP)	3100 (100)	980 (100)	800 (100)	50 (100)
Diethyl fluorophosphate (DEFP)	3800 (120)	900 (90)	600 (75)	100 (200)
Di-*n*-butyl fluorophosphate (DBFP)	2150 (55)	960 (100)	460 (60)	50 (100)
Bis(dimethylamino)fluorophosphate	0 (0)	0 (0)	0 (0)	
Tetraethyl pyrophosphate (TEPP)	1300 (40)	390 (40)	130 (15)	20 (40)
Tetra-*n*-propyl fluorophosphate	2600 (80)	650 (65)		
Tetraisopropyl fluorophosphate	350 (10)	160 (15)		
Tetrakis(dimethylamino)pyrophosphoramide	0 (0)	0 (0)	0 (0)	
O,O-Dimethyl-2,2,2-trichloro-1-hydroxyethyl phosphate (Dipterex)	0 (0)	0 (0)	0 (0)	
Dichlorovinyl dimethyl phosphate (DCVMP)	610 (40)*	250 (50)*	190 (45)*	
Triethyl phosphate	0 (0)	0 (0)	0 (0)	
Diethyl cyanophosphate	320 (10)	230 (25)		
O,O-Diethyl-*O*-4-nitrophenyl phosphate (Paraoxon)	<50	0 (0)	<20	0 (0)
O,O-Diethyl-*O*-4-nitrophenyl thiophosphate (Parathion)	0 (0)	0 (0)	0 (0)	
O,O-Dimethyl-*O*-3-chloro-4-nitrophenyl thiophosphate (Chlorothion)	0 (0)	0 (0)	0 (0)	

* Percentage rate times 2 as hydrolysis of 1 mole of substrate liberates 1 mole of acid (*cf.* Mounter and Dien (20)).

these were incubated for 15 minutes with the organism before mixing with the substrate.

EXPERIMENTAL

P. pentosaceum had not been studied previously with respect to DFP hydrolysis, and the effect of metals on the activity was investigated. It was

found that Co^{++} was an effective activator, whereas Mg^{++} and Mn^{++} showed much less effect. The rate of hydrolysis of DFP by *E. coli* was considerably greater than that reported previously (4) and may be due to the fact that the preparations were obtained from different strains of the organism.

The data for the hydrolysis of a number of organophosphorus compounds by the four species of organisms are presented in Table I. Each figure represents the average of at least four values obtained from separate duplicate determinations. Fluorophosphates, pyrophosphates, and dimethyl-

TABLE II

Effect of Inhibitors on Hydrolysis of Organophosphorus Compounds by Microorganisms

Organism	Substrate	Per cent inhibition by			
		Phenylmercuric chloride (10^{-5} M)	Cu^{++} (3×10^{-5} M)	Ethylenediaminetetraacetic acid (10^{-3} M)	Nitrogen mustard (10^{-4} M)
E. coli	DEFP	15	50	60	40
	DFP	20	40	65	50
	DBFP	25	35	65	50
	TEPP	20	40	50	
	DCVMP	20	45	60	
P. fluorescens	DEFP	40	10	45	10
	DFP	40	10	35	20
	DBFP	30	15	45	10
	TEPP	35	15	40	
	DCVMP	35	10	45	
S. faecalis	DEFP	20	10	50	5
	DFP	20	10	50	15
	DBFP		10	60	10

chlorovinyl phosphate are hydrolyzed at appreciable rates. Bis(dimethylamino)fluorophosphate and tetrakis(dimethylamino)pyrophosphate, which represent compounds in which dimethylamino groups have replaced alkyl radicals, are not hydrolyzed. Dialkylaryl phosphates, dialkylaryl thiophosphates, and simple triesters such as triethyl phosphate do not appear to be hydrolyzed.

The dimethyl ester of oxytrichloroethylenephosphoric acid (Dipterex) (12) is a relatively new insecticide of low mammalian toxicity (13). Upon treatment with dilute alkali, hydrochloric acid is eliminated and the compound undergoes rearrangement to yield dimethyldichlorovinyl phosphate (14, 15), which has considerable toxicity. From the data in Table I, it is seen that Dipterex is not affected, but its chlorovinyl derivative is readily

hydrolyzed. It is worth noting that hog kidney DFPase also hydrolyzes dimethyldichlorovinyl phosphate but not Dipterex.

In specificity studies, it is necessary to prove that a single enzyme is hydrolyzing a number of different substrates. One method for studying such a problem involves the use of inhibitors and the determination of their relative effects with the different substrates. The effect of four inhibitors on DFPase activities of three species of microorganisms is presented in Table II. It is seen that the degree of inhibition of hydrolysis of the substrates is approximately the same for each species.

In view of the structural differences of tetraethyl pyrophosphate, diisopropyl fluorophosphate, Paraoxon, and dimethylchlorovinyl phosphate, the

TABLE III

Hydrolyses of Mixed Substrates by Microorganisms

Substrate concentration, 25 mM. The rate of hydrolysis is given in microliters of CO_2 per ml. of cell suspension per 30 minutes.

Organism	Substrate		Mixture			
	A	B	A	B	A + B	A + B calculated
E. coli	DFP*	DCVMP	560	190	520	750
	TEPP	Paraoxon	220	5	180	225
	DBFP	"	440	0	330	440
P. fluorescens	TEPP	DBFP	350	620	670	970
	DFP	"	450	470	470	920
P. pentosaceum	DFP	DCVMP	170	50	115	220
	DEFP	TEPP	220	40	190	260

* For a list of the abbreviations used, see Table I.

hydrolyses of mixtures of these substances have been determined at high concentration at which the limiting velocity of hydrolysis is being approached (Table III). The rates of hydrolysis of such mixtures were found to be smaller than the sum of the values for the individual substrates. These results are in agreement with the belief that each substrate is competing for the same active centers of a single enzyme.

DISCUSSION

The results obtained in this investigation show that DFPases of microorganisms hydrolyze various types of organophosphorus compounds. The enzymes are activated by metal ions and hydrolyze fluorophosphates and pyrophosphates, but do not attack the dialkylaryl esters studied. The relative rates of hydrolysis of the various substrates differ to some degree among different species of microorganisms and in general are similar to that observed with hog kidney enzyme (16).

Doherty and Vaslow (17, 18) and Doherty (19) found that 4-(*p*-hydroxy)-3-acetaminobutanone-2 is a competitive inhibitor of chymotrypsin, whereas 5-(*p*-hydroxyphenyl)-3-ketovalerate is hydrolyzed by this enzyme. Doherty concluded that bond rupture will take place only when the activation energy can be sufficiently lowered by the binding of the appropriate groups in a compound at the active centers of the enzymes. It has been shown (Table I) that DFPase hydrolyzes dialkyl fluorophosphates, tetraalkyl pyrophosphates, and dialkylchlorovinyl phosphates, but has no effect on the respective analogues, bis(dialkylamino)fluorophosphates, tetrakis(dimethylamino)pyrophosphates, or trialkyl phosphates. These differences are probably derived from the influence of substituents in phosphorus esters on the phosphorus atom. The ability of DFPase to hydrolyze organophosphorus substrates may be attributed to inductive effects of electronegative substituents.

SUMMARY

Enzymes present in microorganisms are capable of hydrolyzing dialkyl fluorophosphates, tetraalkyl pyrophosphates, dimethyldichlorovinyl phosphate, and diethyl cyanophosphate, but not dialkylaryl phosphates.

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INTRACELLULAR PROTEIN METABOLISM IN EHRLICH'S ASCITES CARCINOMA CELLS*†

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Hogness *et al.* (2) have observed that S^{35}-labeled *Escherichia coli* proteins do not significantly contribute to the synthesis of the induced enzyme β-galactosidase. Spiegelman *et al.* (3) have made similar observations with the use of C^{14}. Their results indicate that protein synthesis in these cells is practically irreversible, and suggest that proteins are not in dynamic equilibria with their constituent amino acids. However, numerous reports in the literature cite the loss of isotope from labeled proteins in mammalian tissues and have been interpreted as evidence of the dynamic state of proteins. Rather than imply an essential difference in the metabolism of proteins in mammalian and bacterial cells, Hogness *et al.* (2) have sought an explanation based on the different properties of the two systems. Most mammalian tissues consist of heterogeneous populations of cells under different environments in which some cells are undergoing growth and multiplication while others are dying and lysing; some cells secrete large amounts of protein while others appear to remain stable. Cell lysis and protein secretion would thus afford pathways, other than that involving dynamic equilibrium, for the loss of labeled proteins from mammalian tissues.

It was considered of interest to investigate this aspect of protein metabolism in a mammalian system. Ascites tumor cells, which can be grown and incubated as free cell suspensions, provide a preparation in which a fairly uniform environment can be maintained throughout the entire cell population. Release of proteins from these cells by secretion or as a result of cell lysis is small and is easily determined. The evidence presented in this report supports the hypothesis that proteins exist in dynamic equilibria within the cell.

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EXPERIMENTAL

Preparation of Cells—Ehrlich's mouse ascites carcinoma cells (4) were recovered from the peritoneal cavity of mice 6 to 7 days after the intraperitoneal inoculation with small amounts of tumor. The cells were obtained free from the ascitic fluid by centrifugation and were suspended in 9 volumes of Krebs-Ringer-phosphate buffer solution, pH 7.4. The buffer solution contained 0.2 ml. of ascitic fluid, 30 units of potassium penicillin G, and 30 γ of streptomycin sulfate per ml.

Ascites cells labeled *in vivo* with C^{14} were prepared by single intraperitoneal injections. Approximately 3 mg. of DL-alanine-2-C^{14}, with a specific activity of 4×10^6 c.p.m. per mg. or 2.5 mg. of DL-lysine-2-C^{14} with a specific activity of 2×10^6 c.p.m. per mg. were used per animal. In one group of experiments, 1 mg. of glycine-2-C^{14} with a specific activity of 15×10^6 c.p.m. per mg. was used. The isotopically labeled amino acids were injected 5 days after the inoculation of the mice with the tumor and 2 days prior to the removal of the ascites cells. Only non-hemorrhagic preparations were used. The cells were separated from the ascitic fluid by centrifugation and resuspended in Krebs-Ringer-phosphate buffer solution as described above.

Incubations in Vitro—In the experiments *in vitro* which involve unlabeled or C^{14}-labeled ascites cells freshly removed from tumor-bearing mice, 3 ml. aliquots of the 1:10 ascites cell-buffer solution suspension were incubated in 25 ml. Erlenmeyer flasks at 37° with continuous shaking. The flasks containing unlabeled cells received 500,000 c.p.m. of radioactive alanine, lysine, or glycine. Some of the incubations with cells previously labeled *in vivo* were repeated in bicarbonate buffer in the presence of 3 mg. of glucose per flask, but no significant differences were observed. Incubations *in vitro* were carried out in duplicate for 3, 6, and 9 hours. At the end of the incubation period, the contents of the flasks were centrifuged to obtain the ascites cells. Separation into acid-soluble, lipide, nucleic acid, and protein fractions was carried out after precipitation of the acid-insoluble portion with perchloric acid (5, 6). Values presented for the nucleic acid fraction include both ribose and deoxyribose nucleic acids.

In the experiment with radioglycine, the acid-soluble fraction was further separated into anionic, cationic, and neutral constituents by means of several 1 × 2 cm. ion exchange resin columns. After the removal of the perchlorate as the insoluble potassium salt, the slightly alkaline "acid-soluble" fraction was passed through a Dowex 1 formate column. The effluent and wash were adjusted to pH 2 and passed through a Dowex 50 H^+ column. A 2 N HCl eluate from the Dowex 1 column and a 1 M NH_4OH eluate from the Dowex 50 column were obtained as well as a neutral effluent from both columns (Dowex 50 effluent). Since the Dowex 50 eluate con-

tained small amounts of material which absorbed in the ultraviolet region with a peak at about 260 mμ, further purification was achieved by aerating the ammonia and passing the neutralized solution through an activated charcoal column. Only the values obtained for the charcoal effluent, which should correspond to a purified free amino acid preparation, are included in the present study. Portions of this fraction were also chromatographed on paper with water-saturated phenol as the solvent.

Incubations in Vivo—Samples of 1 ml. of the cell suspension in Krebs-Ringer solution were placed in cellophane dialysis sacs (0.7 × 2 cm.) which were tied at both ends. The bags thus prepared were introduced into the peritoneal cavity of normal mice and allowed to remain for the duration of the *in vivo* "incubations." The dialysis bags were then removed, and the contents quantitatively recovered by washing with small volumes of buffer solution.

In the experiments which involved the implantation of non-radioactive cells, the material recovered from the implants in dialysis bags was suspended in a total volume of 3 ml. with Krebs-Ringer-phosphate buffer solution containing 1.5 mg. of glucose per ml. Incubations *in vitro* were carried out for various periods of time at 37° with continuous shaking in the presence of C^{14}-labeled lysine. Each flask received approximately 250,000 c.p.m. of lysine-2-C^{14}. At the end of the *in vitro* incubations, the proteins were prepared as described above.

In the experiments which involve the implantation of cells previously labeled with radiocarbon, the contents of the bags were recovered and the cells obtained by centrifugation. After precipitation of the acid-insoluble residue with perchloric acid, the lipide, nucleic acid, and protein fractions were prepared (5, 6). All the incubations were carried out in duplicate.

Measurements of Radioactivity—Duplicate aliquots from all fractions were plated directly, and the radioactivity was determined with a gas flow tube. The proteins were plated from a suspension in ethanol-water. A minimum of 4000 counts was taken and the appropriate self-absorption corrections applied.

Results

The incorporation *in vitro* of alanine-2-C^{14}, lysine-2-C^{14}, and glycine-2-C^{14} into the lipides, nucleic acids, and proteins of ascites cells incubated for periods up to 9 hours is shown in Table I. The rate of uptake of all three amino acids is markedly diminished after the 3rd hour of incubation. Incorporation, indicative of metabolic activity, continues up to and beyond the 9th hour.

Table II presents the results obtained from incubations *in vitro* of cells previously labeled with radioactive alanine and lysine. The activity in the

lipide fractions is low originally but increases with time of incubation. The radioactivity associated with the nucleic acids and proteins decreases 12 and 20 per cent, respectively, in 9 hours in the experiments with alanine-labeled cells. Incubation of cells labeled with C^{14}-lysine resulted in a re-

TABLE I

*Incorporation in Vitro of Labeled Alanine, Lysine, and Glycine into Ascites Cells**

The results are expressed as total counts per minute recovered in each fraction.

	Alanine			Lysine			Glycine		
	3 hrs.	6 hrs.	9 hrs.	3 hrs.	6 hrs.	9 hrs.	3 hrs.	6 hrs.	9 hrs.
Lipide	82	244	456	745	810	942	1,700	2,400	2,700
Nucleic acid	4270	5050	5350	722	782	890	19,600	32,200	38,700
Protein	6290	6750	7380	16,700	18,500	19,700	40,700	54,500	60,700

* 500 mg. wet weight of cells were incubated with approximately 500,000 c.p.m. of the C^{14}-labeled amino acid.

TABLE II

Concentration of Radiocarbon in Fractions from C^{14}-Labeled Ascites Cells Incubated in Vitro

Ascites cells are labeled with alanine-2-C^{14} and lysine-2-C^{14}, respectively.

Time of incubation	Radioactivity*						Weight of protein		Specific activity of protein†	
	Lipide		Nucleic acid		Protein					
	Alanine	Lysine	Alanine	Lysine	Alanine	Lysine	Alanine	Lysine	Alanine	Lysine
hrs.							*mg.*	*mg.*		
0	92	477	1630	590	3560	34,400	30.32	35.18	118	980
3	113	483	1550	582	3370	32,200	29.67	33.27	114	960
6	141	574	1440	600	3000	29,200	27.71	32.57	108	890
9	169	694	1400	608	2900	26,100	27.64	32.26	106	810

* Total counts per minute in each fraction.

† Counts per minute per mg. of protein.

lease of 27 per cent of the protein-bound C^{14}. No change was observed in the isotope content of the nucleic acid fraction. Approximately 8 to 10 per cent of the cells incubated is lost through lysis, as reflected by the weight of the protein recovered. Cell counts on sedimentable material, not reported here, confirm this finding. Cell loss thus accounts for most of the radioactivity lost from the nucleic acids, but protein-bound radioactivity is reduced by 10 to 17 per cent in excess of this, as shown by the decrease in the specific activity.

The results of analyses performed on incubations with cells labeled *in vivo* with C^{14}-glycine are presented in Table III. Radioglycine was used since its high specific activity yielded a much greater labeling in the protein than with alanine or lysine. The suspending medium obtained by centrifugation at the end of the incubation period was also analyzed. The acid-soluble fractions from the cells and the medium were processed as described above to obtain a purified "free amino acid" preparation. Table III shows that the radioactivity associated with this fraction rises intracellularly first and then extracellularly during the incubation. The total number of counts recovered account for about 12 per cent of the activity originally present in the protein. Chromatography on paper with H_2O-

TABLE III

*Release in Vitro of Radioactivity from Ascites Cell Proteins Labeled with Glycine-2-C^{14}**

Time of incubation	Charcoal effluent from acid-soluble fractions†		Protein recovered from incubation medium		
	Cells	Incubation medium			Activity‡
hrs.			*mg.*	*c.p.m.*†	
0	2,800	0	3.9	0	0
3	15,000	4400	3.8	6,200	
6	17,600	6000	4.8	8,200	9,100
9	18,700	9400	5.6	16,000	10,100

* The cells in each flask contained about 30 mg. of total protein labeled with approximately 200,000 c.p.m. (specific activity = 6600 c.p.m. per mg.).

† Total counts per minute recovered.

‡ Activity = counts per minute per mg. of additional protein recovered in the extracellular fraction.

saturated phenol revealed only one radioactive area, in the glycine-serine region. Also included in Table III are the values obtained for the protein recovered from the incubation medium. Approximately 4 mg. of protein are present originally from the addition of ascitic fluid. During the 9 hours of incubation, an increase in the extracellular protein is observed which accounts for about 6 per cent of the weight and 8 per cent of the radioactivity of the cell protein. The "activity" of the excess protein found in the incubation medium is 10,000 c.p.m. per mg. as compared to 6600 for the cellular protein.

Analyses of the extracellular protein recovered from the incubations *in vivo* of glycine-labeled cells are summarized in Table IV. Very small amounts of extra protein are recovered in the extracellular medium. The "activity" of the released protein is somewhat higher than the specific activity of the original cell protein.

The effect of the incubation *in vivo* on the growth and multiplication of

the ascites tumor cells is shown in Table V. Within 24 to 48 hours, approximately a 2-fold increase in the number of cells occurs. This observation was made by counting the cells in a hemocytometer counting chamber with a phase-contrast microscope. The cells, on microscopic examination, appeared considerably smaller in the incubated preparations than in the

TABLE IV

*Analysis of Extracellular Protein Recovered from Incubations in Vivo of Dialysis Sacs Containing C^{14}-Glycine-Labeled Ascites Cells**

Time of incubation	Extracellular protein		
		Total c.p.m. recovered	Activity†
hrs.	*mg.*		
0	1.1	0	0
7	1.3	2810	14,100
24	1.6	5030	10,000
48	1.5	5390	13,500

* The cells in each sac contained about 16 mg. of total protein labeled with approximately 130,000 c.p.m. (specific activity = 8100 c.p.m. per mg.).

† Activity = counts per minute per mg. of additional protein recovered in the extracellular fraction.

TABLE V

*Effect of Incubation in Vivo on Multiplication and Growth of Ascites Tumor Cells**

Time of incubation	Experiment A		Experiment B		Experiment C	
	Cells per ml. suspension	Dry weight	Cells per ml. suspension	Dry weight	Cells per ml. suspension	Dry weight
hrs.	$\times 10^{-6}$	*mg.*	$\times 10^{-6}$	*mg.*	$\times 10^{-6}$	*mg.*
0	5.0	26.0	3.6	18.3	5.7	30.3
24	11.6	24.6	6.2	16.7	10.1	32.9
48			7.7	20.4	10.3	29.5

* Intraperitoneal implants of 1 ml. of ascites cell suspension in dialysis membranes.

original samples. Dry weight determinations, however, indicate that no gain in the total dry weight of the cells occurred within the dialysis membrane. The results described in Table V are borne out by the weights of the proteins recovered from implantations of labeled cells (Table VI).

The extent of incorporation of C^{14}-lysine into the proteins of ascites tumor cells which had previously been maintained intraperitoneally in dialysis bags for 24, 48, and 96 hours is shown in Fig. 1. Incubations *in vitro* in the presence of lysine-2-C^{14} were carried out for 1, 2, and 4 hours

with the unlabeled cell suspensions recovered from the membranes. For purposes of comparison, values are included on the incorporation of the amino acid into the proteins of cells maintained for periods of time equivalent to those used in the peritoneal implants, but kept at 4°. Fig. 1 shows

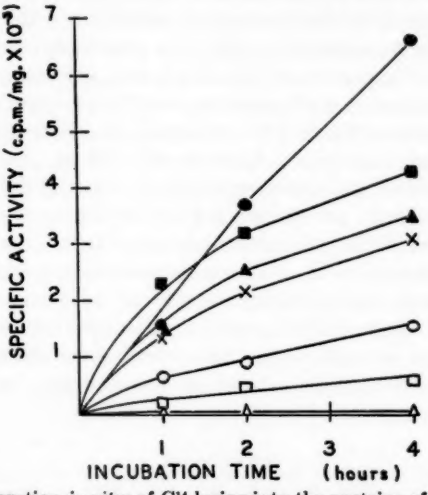

FIG. 1. Incorporation *in vitro* of C^{14}-lysine into the proteins of ascites cells maintained intraperitoneally in dialysis bags. ●, 24 hours; ■, 48 hours; ▲, 96 hours; stored at 4°, ○, 24 hours; □, 48 hours; △, 96 hours; ×, into fresh cells.

TABLE VI

Concentration of Radiocarbon in Fractions from C^{14}-Labeled Ascites Cells Incubated in Vivo

Ascites cells are labeled with alanine-2-C^{14} and lysine-2-C^{14}, respectively.

Time of incubation	Radioactivity*						Weight of protein		Specific activity of protein†	
	Lipide		Nucleic acid		Protein		Alanine	Lysine	Alanine	Lysine
	Alanine	Lysine	Alanine	Lysine	Alanine	Lysine				
hrs.							*mg.*	*mg.*		
0	31	132	505	226	1190	11,400	8.98	12.31	132	920
7	44	207	446	227	1160	8,820	10.97	12.08	106	730
24	51	362	372	197	875	7,650	10.51	12.29	83	620
48	43	570	315	175	809	5,500	10.11	11.60	80	470

* Total counts per minute in each fraction.

† Counts per minute per mg. of protein.

that the activity of reimplanted cells, as reflected by the incorporation of C^{14} from lysine into the proteins, is considerably higher than that of ascites cells freshly removed from tumor-bearing mice. This activity is highest 24 hours after reimplantation in dialysis membranes, decreases with time, and approaches that of fresh cells after 96 hours. Cells stored at 4° exhibit a decreasing ability to incorporate amino acids, the specific activity of the proteins being extremely low in cells previously stored for 96 hours.

Intraperitoneal implantation of cells previously labeled *in vivo* with alanine-2-C^{14} resulted in a decrease in the C^{14} content of the nucleic acid and protein fractions (Table VI). The activity in the lipide fraction appeared to increase with time. Approximately 38 per cent of the radioactivity associated with the nucleic acids was lost during the 48 hour incubation. The decrease in the specific activity of the protein indicated that about 40 per cent of the protein-bound radioactivity was released during this period. The results obtained from comparable experiments performed with cells previously labeled with lysine-C^{14} are also presented in Table VI. The C^{14} activity in the lipide fraction apparently increased in the course of the experiment, but the nucleic acid fraction lost about 22 per cent of its activity and the specific activity of the proteins decreased approximately 50 per cent.

DISCUSSION

The *in vitro* system consists primarily of surviving cell populations. The ability of such preparations to incorporate amino acids is markedly diminished after the first 3 hours. Intraperitoneal implantations of dialysis membranes containing ascites tumor cell suspensions result in increases in the numbers of cells. Concomitant with cellular division is the enhanced ability to incorporate radioactive lysine. Christensen has described briefly the technique of reimplantation of cells in collodion bags (7). The data presented in this report confirm his observation that a 2-fold increase in the number of cells occurs within 48 hours. The finding that a gain in the dry weight of the cells, and therefore chemical growth, did not occur has also been confirmed. Christensen postulates, as possible explanations for the failure of growth, the nutritional requirements of the ascites tumor cell for macromolecules or the exclusion of a possible host factor which ordinarily serves to prevent cohesion of cells observed in his experiments.

Although incubation of C^{14}-labeled ascites cells *in vitro* or *in vivo* resulted in a decrease in the amount of radioactivity associated with the proteins, the lipide fraction continued to incorporate isotope. It is possible that the cell may be utilizing metabolic products of the amino acids released from the protein for the synthesis of lipide components.

In the incubations *in vitro*, no changes were observed in the nucleic acid

fraction with either alanine or lysine-labeled cells. These data would indicate that the nucleic acids may be metabolically inert under these conditions. The labeling of the various components in the ascites cells with either alanine or lysine resulted in the loss of a considerable portion of the radioactivity in the nucleic acid fraction on reimplantation (Table VI). The results obtained with the nucleic acids indicate a difference in the metabolic activity of the two systems investigated, particularly that associated with cell division in the incubations *in vivo*.

In both the *in vitro* and *in vivo* conditions, radiocarbon is released from the protein fraction. A decrease of 10 to 17 per cent in the specific activity of the protein was observed in the 9 hours during which labeled cells were maintained *in vitro*. Intraperitoneal implantation of labeled cells, however, resulted in a decrease of 40 to 50 per cent in the specific activity of the protein in 48 hours. The observation that loss of C^{14} from the proteins occurs without concomitant loss in the amount of protein suggests that radioactive amino acids in the protein are being replaced by non-isotopic constituents from the free amino acid pool. Since the labeled cells used in these experiments were obtained 48 hours after the administration of the isotope, the intracellular pool provides a source of unlabeled free amino acids. In the *in vitro* system, this pool is supplemented by the additions of unlabeled ascitic fluid.

Cowie *et al.* (8) failed to observe a significant release of radioactive sulfur from labeled *E. coli* cells; this observation has been interpreted as further evidence that bacterial proteins are not in a dynamic state. However, ascites cells whose proteins were labeled *in vivo* with radioglycine released free amino acids intracellularly on incubation *in vitro*. Simpson (9) observed that conditions which limit the release or utilization of energy depress the liberation of labeled methionine or leucine from the protein of liver slices *in vitro*. It has been demonstrated that components also exist in yeast cells which can break down to their constituent amino acids (3).

Evidence that protein metabolism is not a one way process in which only synthesis occurs has been presented by a number of investigators. Greenless and LePage (10) and Babson and Winnick (11) have found that radioactivity is released from labeled proteins of actively growing tumor cells. Chibnall (12) has found that N^{15}-ammonia was incorporated into the proteins of detached leaves even while a large amount of protein was being broken down. This finding was presented as evidence of a protein cycle in which protein synthesis is maintained even under conditions which enhance breakdown. Since loss of protein may be occurring through cell lysis, protein secretion, etc., as suggested by Hogness *et al.* (2), it is difficult to interpret these results as direct evidence of an intracellular protein cycle.

The possibility exists that the decrease in the specific activity of the cell

proteins with time results from the preferential leakage from the cell of a small amount of protein of high specific activity. The observations made on the release of C^{14} from glycine-labeled cells do not appear to support this possibility. Free amino acids are released *in vitro* from labeled cells. This release occurs primarily intracellularly. The extent to which free amino acids are released is of the same order of magnitude as the decrease in the specific activity of the protein (12 per cent). The per cent of radioactive protein which appears in the incubation media *in vitro* is in agreement with the per cent of cell loss due to lysis (8 per cent). The total counts per minute and "activity" of the proteins released either *in vivo* or *in vitro* do not appear to be sufficiently high to indicate the preferential loss of a highly labeled protein.

The evidence presented in this study suggests that the decrease in the specific activity of the protein and the appearance of labeled free amino acids within the cell are a reflection of a dynamic process in these cells whereby labeled amino acids in the protein molecule are being replaced by constituents from the free amino acid pool.

SUMMARY

Ehrlich's ascites carcinoma cells were labeled *in vivo* with radioactive alanine, lysine, or glycine.

On incubation *in vitro* of labeled cells, a loss in the radioactivity associated with the proteins occurred. Intracellular release of radioactive free amino acids from labeled proteins was also demonstrated.

Implantation of ascites cells in dialysis membranes into the peritoneal cavity of mice resulted in a 2-fold increase in the number of cells within 48 hours. Growth, as manifested by a gain in weight, did not occur in such preparations. Reimplantation of cells previously labeled with C^{14}-alanine or lysine resulted in a loss of radioactivity from the nucleic acids. The specific activity of the proteins was markedly decreased in the 48 hour period.

The evidence presented suggests that a dynamic equilibrium exists between proteins and free amino acids within these cells.

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CORRECTIONS

On page 22, line 37, and on page 24, line 21, Vol. 219, No. 1, March, 1956, read *3-O-(β-D-Δ^{4,5}-glucoseenpyranosyluronic acid)-2-deoxy-2-acetamido-D-glucose* for *3-O-(β-D-Δ^{3,4}-glucoseenpyranosyluronic acid)-2-deoxy-2-acetamido-D-glucose.*

On page 911, legend to Fig. 8, line 2, and on page 912, legend to Fig. 9, line 2, Vol. 219, No. 2, April, 1956, read *fractions represented in Fig. 7* for *fractions represented in Fig. 8.*

ON THE OCCURRENCE OF GLYCINAMIDE RIBOTIDE AND ITS FORMYL DERIVATIVE*

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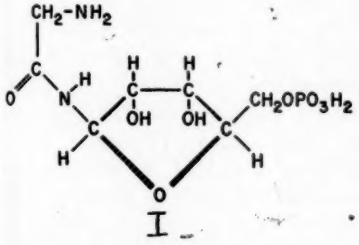
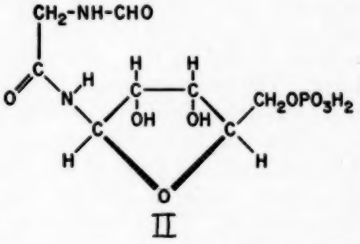
Inosinic acid is synthesized from small molecules such as glycine, formate, and CO_2 by a multienzyme system present in pigeon liver (1–3). This paper reports the occurrence of two new aliphatic ribotides synthesized by this system and describes their isolation and metabolic interrelationships. These compounds have been shown to be precursors of inosinic acid (4–6) and have been assigned the following tentative structures: *N*-glycyl-5-phosphoribofuranosylamine (glycinamide ribotide) (I) and *N*(α-*N*-formyl)glycyl-5-phosphoribofuranosylamine (formylglycinamide ribotide) (II).



I



II

Evidence for the structure of these compounds will be presented in a subsequent paper (7).

Materials

Most of the substrates employed have been described previously (3). Glycine-1-C^{14} was prepared by the method of Sakami *et al.* (8). For radio-

* This work has been supported by grants from Eli Lilly and Company, the Elisabeth Severance Prentiss Foundation, and the National Institutes of Health, United States Public Health Service, grant No. 8-3840.

† Oglebay Fellow in Medicine and Biochemistry, 1952–55.

‡ DuPont Predoctoral Fellow, 1954–55. A portion of this work is included in the thesis submitted by Richard A. Peabody to the Graduate School of Western Reserve University in September, 1955, in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

XUM

autographic studies a specific activity of 77,000 c.p.m. per μmole was employed, while for isolation of intermediates on ion exchange columns the activity was 3000 to 5000 c.p.m. per μmole.

The preparation of the fresh acetone powder extract has been described (3). The Dowex-treated and dialyzed extract was prepared by extracting 10 gm. of acetone powder (3) with 100 ml. of 0.05 M K_2HPO_4 for 30 minutes. This material was centrifuged at 5000 × *g* for 20 minutes, and the supernatant solution was passed through a Dowex 1 column (HCO_3^- form, 1.82 sq. cm. × 12 cm.) for a period of 1 to 2 hours. The column was washed with 20 ml. of water, and the combined effluents were dialyzed against running 0.05 M K_2HPO_4 solution (20 liters) for 18 to 24 hours and lyophilized (yield 3.4 gm.). All operations prior to lyophilization were performed at 0–4°. The powder was taken up in water (100 mg. per ml.) immediately before use. The protein concentration of this solution was 70 to 80 mg. per ml.

Methods

Determination of C^{14}-Formate Incorporation into Purine and Non-Purine Fractions—The C^{14}-formate incorporated into the 2 and 8 carbons of inosinic acid and hypoxanthine was determined by hydrolysis with sulfuric acid, oxidation of the resulting CO to CO_2, and measurement of the radioactivity as $BaCO_3$ (3). The results were corrected to an infinitely thin layer. The incorporation of formate into all fractions (total formate fixation) was determined by pipetting a 0.05 ml. aliquot of the trichloroacetic acid filtrate of the reaction mixture into a glass planchet (Tracerlab, Inc.). After addition of 5 drops of water and 5 drops of alcohol, the solution was dried slowly under an infra-red lamp, and the radioactivity of the residue was measured. These results were corrected to give values equivalent to those obtained when the samples were counted as $BaCO_3$. The non-purine fraction was calculated as the difference between the total formate fixed per vessel and the formate incorporated into the 2 and 8 carbons of purine.

Assay for Formylglycinamide Ribotide—The synthesis of formylglycinamide ribotide was measured by the incorporation of C^{14}-formate into a form which was unstable to acid hydrolysis. This was calculated as the difference between the total formate fixation (described above) and the fixation into a fraction stable to acid hydrolysis. To determine the radioactive fraction not hydrolyzed by acid, 0.2 ml. of the trichloroacetic acid filtrate was heated at 100° for 15 minutes with 0.1 ml. of 3 N HCl. After dilution with water to 2.0 ml., a 0.5 ml. aliquot was pipetted into a glass planchet, dried, and counted.

Assay for Glycinamide Ribotide—The formation of the glycinamide ribotide was estimated by employing glycine-1-C^{14} as a precursor. The amide

linkage in glycinamide ribotide and in formylglycinamide ribotide was not affected by heating to 100° at pH 5.4. Therefore, on treatment of an aliquot of the reaction filtrate with ninhydrin, the radioactivity which remained after the residual glycine-1-C^{14} was decarboxylated represented glycinamide ribotide (or formylglycinamide ribotide). A 0.1 ml. aliquot of the trichloroacetic acid filtrate was pipetted carefully into the bottom of a small test-tube and neutralized with 1 N sodium hydroxide by using brom thymol blue. 1 ml. of 0.1 M potassium phosphate buffer, pH 5.4, 0.1 ml. of 0.1 M non-radioactive glycine, and 1.0 ml. of ninhydrin solution (30 mg. per ml.) were added. The tube was covered with a glass marble and heated at 100° for 30 minutes. The mixture was cooled, and after the addition of 1 drop of caprylic alcohol it was aerated with CO_2 for 15 minutes. Water was added to 10 ml., and a 2 ml. aliquot was pipetted into a glass planchet, dried, and counted. The factor for the correction of the results to an infinitely thin layer in a glass planchet was found to be approximately 2.4. Evidence for the specificity of these assays is presented under "Results."

Chromatography—For all paper chromatographic studies except the large scale preparations, Whatman No. 1 paper was employed. Two-dimensional chromatograms were developed by the ascending technique. For large scale preparations a thick paper (Eaton and Diekman No. 627-030) was used. A single thickness of Whatman No. 1 was sewn on as a leader to control the rate of solvent flow from the trough to the starting line. The following solvent systems were employed (all ratios are in volume proportions): (1) propanol and water (65:34) in an atmosphere of ammonia (0.5 ml. of 1 M ammonium hydroxide per liter of jar volume), (2) pyridine and water (65:35), (3) *n*-butanol-17.6 N acetic acid-water (2:1:1), (4) isopropanol-water (70:30) with 0.35 ml. of concentrated ammonium hydroxide per liter of jar volume, (5) propanol-water (60:40), (6) 95 per cent ethanol-water (77:23), and (7) methanol-formic acid (88 per cent by weight)-water (80:15:5). With Solvent 6 the paper was dipped in 1.2 per cent sodium citrate buffer, pH 5.4, and dried prior to application of compounds.

Radioactive compounds were located with a monitor and were eluted with water from the papers by descending chromatography. For radioautograms, precursors with a specific activity of 60,000 to 120,000 c.p.m. per μmole were employed. 0.1 ml. of the trichloroacetic acid reaction filtrate was applied to a spot 1 cm. in diameter and was chromatographed in two dimensions. The dried chromatogram was exposed to x-ray film for 4 to 7 days. A permanent photographic record of a film occasionally was made on Kodagraph Contact Standard paper.

For anion exchange chromatography Dowex 1 (4 per cent cross-linked, 250 to 400 mesh, formate form) was employed. Reaction mixtures were

adjusted to pH 9.0 with ammonium hydroxide solution and applied to the column.

Results

Incorporation of C^{14}-Formate and Glycine-1-C^{14} into Unidentified Fractions —Fig. 1 shows the incorporation of C^{14}-formate into the purine and non-purine fractions at different time intervals. The total C^{14}-formate in the non-purine fraction increased to a maximum at 40 minutes, while the total activity in the purine fraction increased during the 120 minute period. This suggested a precursor-product relationship.

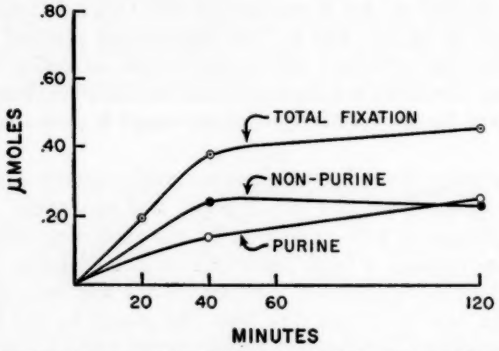


FIG. 1. The rate of incorporation of C^{14}-formate into purine and non-purine fractions. The reaction system was similar to that described under Table I.

In Table I the effect of bicarbonate and boiled extract on the incorporation of C^{14}-formate into the purine and non-purine fractions is presented. When bicarbonate was omitted, the non-purine fraction decreased from 0.22 to 0.14 μmole, while the purine fraction decreased from 0.14 to 0.03 μmole. This resulted in a shift of the non-purine to purine ratio from a value of 1.6 to 4.7. While in this experiment a fall in the non-purine fraction occurred when bicarbonate was omitted, in others an increase was observed. Omission of the boiled extract appeared to decrease purine synthesis and to have no significant effect on the non-purine fraction. It should be pointed out that the extract used in these experiments had not been treated with Dowex and had not been dialyzed.

The rate of fixation of glycine-1-C^{14} was found to be proportional to time (Table II) for 90 minutes. After 30 minutes the total C^{14}-glycine fixation exceeded purine synthesis by a factor of 5.6. This indicated that C^{14}-glycine as well as C^{14}-formate (Table I) was incorporated into compounds

other than inosinic acid and hypoxanthine. In Table II[1] purine synthesis was measured by the incorporation of $C^{14}O_2$ into carbon 6 (3). $C^{14}O_2$ was not significantly diluted by endogenous carbon in such experiments.[2]

TABLE I

C^{14}-Formate Incorporated into Carbons 2 and 8 of Total Hypoxanthine Fractions and into Non-Purine Fraction

Omissions	Purine (C2 + C8) (*a*)	Non-purine (*b*)	$\frac{(b)}{(a)}$
	μmole	*μmole*	
None	0.14	0.22	1.6
$KHCO_3$	0.03	0.14	4.7
Boiled extract	0.09	0.20	2.2

The reaction mixture contained 0.4 ml. of an extract of pigeon liver acetone powder, 1.0 μmole of IMP, 5 μmoles of ATP, 15 μmoles of PGA, 7 μmoles of $KHCO_3$, 8 μmoles of $MgCl_2$, 16 μmoles of DL-homocysteine, 0.2 ml. of boiled extract of pigeon liver (3), and 5 μmoles of C^{14}-formate in a final volume of 0.65 ml. The mixture was incubated 40 minutes at 38° in air.

TABLE II

Total Glycine "Fixation" and De Novo Synthesis of Purine

Time	Glycine-1-C^{14} incorporation	$C^{14}O_2$ incorporation into purine
min.	*μmoles*	*μmole*
15	0.22	
30	0.42	0.075*
60	1.10	
90	1.60	

The reaction mixture contained 0.5 ml. of acetone powder extract, 5 μmoles of glycine-1-C^{14}, 5 μmoles of sodium formate, 10 μmoles of glutamine, 50 μmoles of $KHCO_3$, 1.2 μmoles of ATP, 14 μmoles of PGA, 5 μmoles of $MgCl_2$, 5 μmoles of DL-homocysteine, and 5.5 μmoles of KH_2PO_4 in a final volume of 1.3 ml. The mixture was incubated at 38° in air.

* In this vessel 50 μmoles of $KHC^{14}O_3$ and 10 μmoles of non-radioactive glycine were used. The C^{14} activity of carbon 6 of the purine fraction was determined (3).

Radioautographic Observations on Accumulation of Glycinamide Ribotides—Fig. 2 is a radioautogram of a typical two-dimensional chromatogram. It demonstrates the occurrence of a number of compounds which are la-

[1] The abbreviations used are: ATP, adenosine triphosphate; IMP, inosine-5′-phosphate; AMP, adenosine-5′-phosphate; PGA, 3-phosphoglycerate; R5P, ribose-5-phosphate; CF, calcium leucovorin.

[2] Goldthwait, D. A., unpublished data.

beled by glycine-1-C^{14}. The area *A* denotes the location of glycinamide ribotide and formylglycinamide ribotide, *B* the position of glycine, and *C* the position of hypoxanthine. The identities of the less dense spots are not known. In a comparable experiment, C^{14}-glycine was added to one vessel and C^{14}-formate to another. Fig. 3 shows radioautograms of chromatograms of the two reaction filtrates. Some of the compounds labeled by C^{14}-glycine and some labeled by C^{14}-formate have similar chromato-

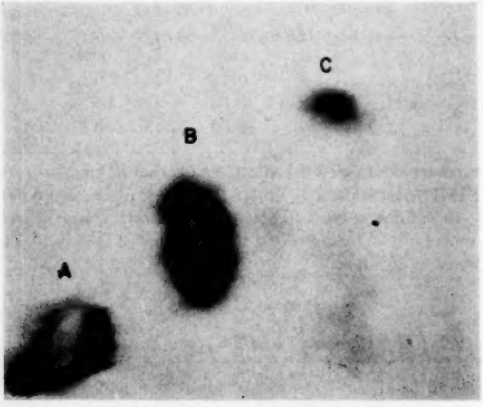


FIG. 2. Incorporation of glycine-1-C^{14} into various compounds. Area *A* represents nucleotides, *B* glycine-1-C^{14}, and *C* hypoxanthine. The reaction mixture contained 0.5 ml. of acetone powder extract, 5 μmoles of glycine-1-C^{14}, 10 μmoles of sodium formate, 50 μmoles of $KHCO_3$, 10 μmoles of glutamine, 5.0 μmoles of ATP, 10 μmoles of PGA, 8 μmoles of $MgCl_2$, and 0.2 ml. of boiled extract of liver in a final volume of 1.1 ml. The mixture was incubated for 2 hours at 38° under N_2. Then 0.25 ml. of 20 per cent trichloroacetic acid was added, and 0.1 ml. of the filtrate was placed on the lower left corner of the paper and chromatographed in the vertical direction with solvent System 1 and in the horizontal direction with solvent System 2.

graphic behavior; compare the spots marked *A*. Fig. 4 provides evidence that the radioactive compounds in *A* of Figs. 2 and 3 were not compounds known to incorporate C^{14}-formate or glycine-1-C^{14}. Fig. 4 is a reproduction of a chromatogram of a reaction filtrate to which methionine, cystathionine, glycine, serine, hypoxanthine, inosine, and inosinic acid were added as marker substances. When $HC^{14}O_3^-$ was used as a tracer under the same conditions and the reaction filtrate was chromatographed, no radioactivity was detected in the spot comparable to Fig. 2, *A*.

A preliminary experiment was carried out to determine whether these compounds were purine precursors. A portion of the compounds in *A* labeled with C^{14}-glycine was found to be converted to inosinic acid in the

presence of a large pool of unlabeled glycine. More detailed experiments on the incorporation of glycinamide ribotide and formylglycinamide ribotide have been published (6).

Isolation of Glycinamide Ribotide and Formylglycinamide Ribotide—Inorganic phosphate was separated from the ribotide fraction by chromatography of the reaction filtrates on thick paper (solvent System 3). An alternative procedure involved the removal of the trichloroacetic acid by

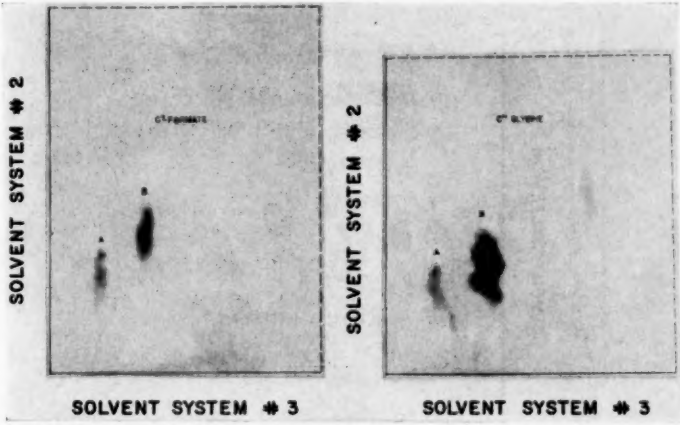


FIG. 3. The incorporation of glycine-1-C^{14} and C^{14}-formate into comparable compounds. Area *A* represents glycinamide ribotides, and area *B*, serine and glycine. The reaction mixture contained 0.5 ml. of extract of acetone powder, 5 μmoles of glycine or glycine-1-C^{14}, 7.5 μmoles of sodium formate or C^{14}-formate, 37 μmoles of $KHCO_3$, 10 μmoles of glutamine, 2 μmoles of ATP, 7 μmoles of PGA, 8 μmoles of $MgCl_2$, 5 μmoles of DL-homocysteine, and 0.2 ml. of boiled extract in a final volume of 1.2 ml. The mixture was incubated at 38° for 2 hours. 0.3 ml. of 20 per cent trichloroacetic acid was added and 0.1 ml. of the reaction filtrate was placed on the lower left corner of the paper and chromatographed. Solvent System 2 (see "Methods") was used for the vertical development and solvent System 3 for the horizontal.

extraction with ether and the precipitation at pH 8.0 of inorganic phosphate and some phosphate esters by addition of barium hydroxide solution. The glycine ribotides remained in the supernatant solution. In preliminary large scale preparations of the glycinamide ribotides, glycine-1-C^{14} was incubated with undialyzed extract of acetone powder and with additions as described under Fig. 5. The entire trichloroacetic acid filtrate was chromatographed on thick paper, and the radioactive compounds which migrated with AMP and IMP were eluted, made alkaline with ammonium hydroxide, and placed on a Dowex 1 formate column. Fig. 5 rep-

resents a typical elution pattern. With the large column employed in this experiment (2.0 sq. cm. × 32 cm.), glycinamide ribotide (I) was eluted with 0.05 M ammonium formate, pH 5.2. Under these conditions, two radioactive bands appeared. However, with a small column (0.64 sq. cm. × 8 cm.) the compound was eluted with buffer of the same molarity at pH 6.5 and, in this case, did not separate into the two components. With the large column formylglycinamide ribotide (II) was eluted at pH 5.0 and was separated into two components.

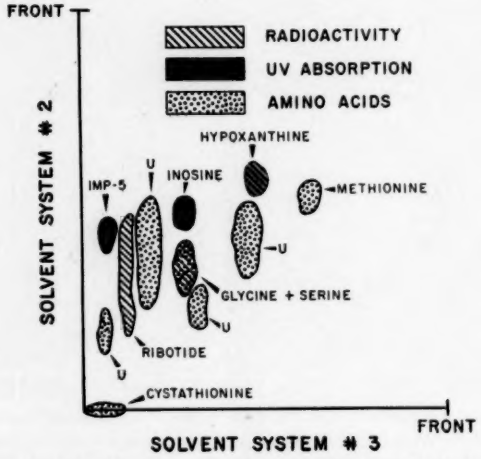


FIG. 4. Comparison of the paper chromatographic behavior of the aliphatic ribotides with other compounds. U represents unidentified compounds which reacted with ninhydrin.

The quantity of glycinamide ribotide varied in different experiments from 6 to 42 per cent of the total aliphatic ribotides. The factors responsible for these variations in the crude extracts are not yet clear. The first component of glycinamide ribotide eluted from the column was consistently larger than the second, while the first component of the formylglycinamide ribotide accounted for 29 to 49 per cent of its total. Elution of the Dowex 1 column at pH 9 (Fig. 5) yielded glycine and a compound which was readily decarboxylated with ninhydrin and which was not a precursor of the purine ring.

Isomeric Nature of Two Components of Each Ribotide—The evidence that these components (Fig. 5) were isomers was as follows: By exposure to acid, it was demonstrated that each of the components of formylglycinamide ribotide could be converted to a mixture of the two. Thus, when

the first component was exposed to 5 per cent trichloroacetic acid at 2° for 24 hours and then rechromatographed, two components appeared. The elution pattern was similar to that in Fig. 5 except that the material in the first peak accounted for 68 per cent of the total. Under comparable acid conditions the second component yielded two peaks with 46 per cent of the total material in the first. As a control the second component was allowed to stand at pH 7.0 at 2° for 48 hours. When this was passed through the column, only a single component appeared. This chromato-

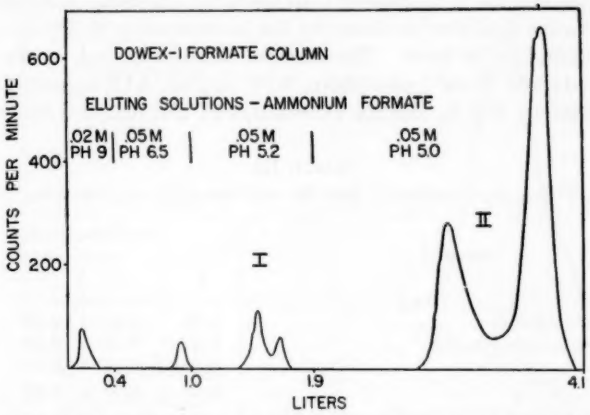


FIG. 5. Elution of glycinamide ribotide (I) and formylglycinamide ribotide (II) from a Dowex 1 column. The reaction mixture contained 12 gm. of a lyophilized extract of acetone powder, 1.0 mmole of glycine-1-C^{14} (3×10^6 c.p.m.), 2.0 mmoles of sodium formate, 0.4 mmole of IMP, 2.0 mmoles of glutamine, 0.5 mmole of ATP, 2.8 mmoles of PGA, 1.6 mmoles of $MgCl_2$, 1.0 mmole of DL-homocysteine, and 47 ml. of boiled extract in a final volume of 240 ml. The reaction mixture was incubated at 38° for 1.5 hours under N_2.

graphic behavior following exposure to acid as well as the analytical data on the two components of formylglycinamide ribotide (5, 6) suggested that isomers existed. Analyses of the two purified components of glycinamide ribotide (7) and of its formyl derivative (5, 6) lend further support to the suggestion that both ribotides can exist as isomers. There is no evidence that these isomers exist in nature.

Some Chromatographic Properties of Glycinamide Ribotides—The behavior of these compounds on Dowex 1 (formate form) is illustrated in Figs. 5 and 6. Formylglycinamide ribotide was not retained on a Dowex 50 (H^+ form) column at pH 1.0. It was immediately eluted from a charcoal column with 5 per cent aqueous ethanol. In most of the solvent systems both of the aliphatic ribotides migrated on paper chromatograms with the

mononucleotides. R_F values with several solvents are presented in Table III.

Cofactor Requirement for Formylation of Glycinamide Ribotide—It was considered that the formylation of glycinamide ribotide required a folic acid derivative, as had been demonstrated for the conversion of 5-amino-4-imidazolecarboxamide-5′-phosphoriboside to inosinic acid (9, 10).

Fig. 6 illustrates the effect of the cofactor upon the synthesis of the aliphatic ribotides. The natural formylation cofactor was removed by treatment of the enzyme with Dowex 1, followed by dialysis. A reduced folic acid derivative then was necessary for the incorporation of C^{14}-formate into an acid-hydrolyzable form. The reaction mixture in each of the experiments contained ribose-5-phosphate, ATP and an ATP-regenerating system, glutamine, $MgCl_2$, and the Dowex-treated and dialyzed enzyme. In

TABLE III

R_F Values for Glycinamide Ribotide and Formylglycinamide Ribotide

Compound	R_F in solvent system*			
	4	5	6	7
Glycinamide ribotide	0.64	0.57	0.50	0.50
Formylglycinamide ribotide	0.24	0.33	0.14	0.46
IMP	0.13	0.28	0.18	0.21
Glycine	0.49	0.36	0.27	0.60

* Solvent systems (see "Methods").

Experiment 1 glycine-1-C^{14} was employed as a tracer. Experiment 2 differed from Experiment 1 only by the addition of calcium leucovorin (DL mixture of CF) and of unlabeled sodium formate. In Experiment 3, C^{14}-formate was employed as the tracer, and unlabeled glycine and CF were added. At the end of the incubation the mixtures were treated as described in the legend to Fig. 6 and passed through Dowex formate columns.

Each of the columns was eluted with ammonium formate buffers as in Fig. 5. In Experiment 1 the glycinamide ribotide fraction accounted for all the fixed glycine. In Experiment 2, 8 per cent of the total glycine fixed appeared in a component eluted at pH 6.5. The remainder represented formylglycinamide ribotide. In Experiment 3 formylglycinamide ribotide accounted for 98 per cent of the acid-hydrolyzable C^{14}-formyl compounds. In order to compare the migration of each of the major components, they were rechromatographed on new Dowex 1 columns (formate form, 0.64 sq. cm. × 8.0 cm.). Fig. 6 shows the elution patterns and demonstrates clearly that, in the absence of the folic acid derivative and formate, glycin-

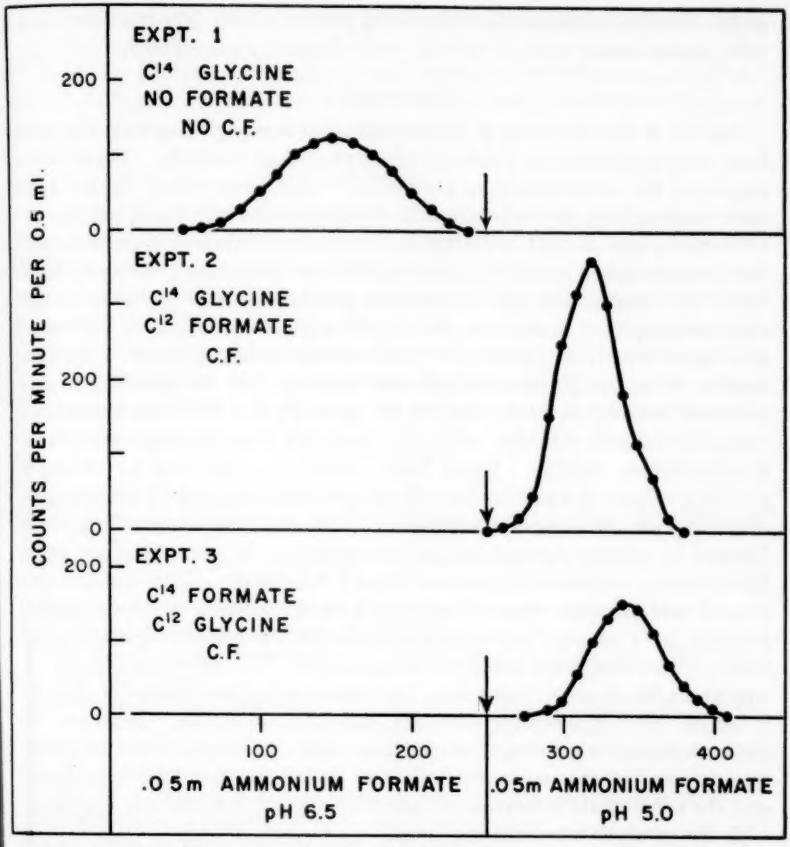


FIG. 6. Effect of citrovorum factor and formate on the synthesis of glycinamide ribotide and formylglycinamide ribotide. The reaction mixtures were prepared by the addition of 200 mg. of lyophilized Dowex-treated and dialyzed extract, 30 μmoles of ATP, 64 μmoles of $MgCl_2$, 50 μmoles of sodium phosphocreatine, 0.5 ml. of a dialyzed 1:2 water extract of rabbit muscle (creatine kinase), 50 μmoles of glutamine, 25 μmoles of R5P, 25 μmoles of C^{14}-formate (47,300 c.p.m. per μmole) or unlabeled formate, 50 μmoles of glycine-1-C^{14} (19,000 c.p.m. per μmole) or unlabeled glycine, and 2 mg. of calcium leucovorin in a final volume of 6.7 ml. Three vessels were prepared identically except in the additions of glycine, formate, and calcium leucovorin indicated. The vessels were incubated 40 minutes at 38° in air. The radioactive products in the trichloroacetic acid filtrates were separated on three identical Dowex 1 columns (formate form 0.64 sq. cm. × 24 cm.) as described in the text. The major component from each column was rechromatographed on a second Dowex 1 column (formate form, 0.64 sq. cm. × 8.0 cm.) as shown in Fig. 6.

amide ribotide accumulated, while in its presence formylglycinamide ribotide, labeled either with C^{14}-glycine or C^{14}-formate, was formed.

DISCUSSION

Studies of the synthesis of glycinamide and formylglycinamide ribotide have been facilitated by relatively rapid analytical methods. These have employed the incorporation of glycine-1-C^{14} into forms which did not lose their radioactivity by reaction with ninhydrin and the incorporation of C^{14}-formate into an acid-hydrolyzable derivative. While it is evident that these methods lack specificity, their validity in these experiments has been tested by a comparison with the reaction products isolated by ion exchange chromatography. In the absence of a folic acid derivative, all of the radioactivity derived from glycine-1-C^{14} and not lost by reaction with ninhydrin was found in the glycinamide ribotide fraction. In the presence of the folic acid derivative, 92 per cent of the fixed glycine-1-C^{14} was present as formylglycinamide ribotide, while the remaining 8 per cent appeared to be in glycinamide ribotide. Under these conditions, there was no evidence for the synthesis of a peptide such as glutathione which would contain glycine in a form not affected by ninhydrin. The formylglycinamide ribotide isolated by column chromatography accounted for 98 per cent of the acid-hydrolyzable radioactivity derived from C^{14}-formate. With the Dowex-treated and dialyzed extract, the total formate fixation was occasionally as much as 15 per cent higher than formate fixation into acid-hydrolyzable forms. This was found mainly in inosinic acid. Incorporation into serine was negligible in most instances. An exception to this appears in Fig. 3, in which area *B* coincides with the migration of serine. However, in these experiments untreated extract was used. No methionine synthesis was detected in the incubation mixtures under the described conditions, and the C^{14}-formate incorporated into folic acid derivatives was negligible with the catalytic amounts employed.

The behavior of these ribotides on an anion exchange column is a reflection of their net charge. Glycinamide ribotide and formylglycinamide ribotide were eluted from the Dowex 1 column with 0.05 M ammonium formate at pH 6.5 and 5.0, respectively. Unlike nucleotides, these compounds can be eluted while they still possess 1 negative charge. This behavior may be due to their aliphatic nature. Ribose-5-phosphate was eluted at pH 5.0 with the formylglycinamide ribotide.

The natural folic acid derivative involved in the formylation of glycinamide ribotide has not been identified. In these experiments the reduced folic acid compound employed represents a model system. The mechanism of formylation is most likely comparable to that involved in the intro-

duction of formate into 5-amino-4-imidazolecarboxamide ribotide to form inosinic acid (10).

Recently Hartman, Levenberg, and Buchanan (11) have reported the accumulation of glycinamide ribotide and the formyl derivative in pigeon liver extracts in the presence of azaserine.

SUMMARY

Glycinamide ribotide and formylglycinamide ribotide, which are precursors of inosinic acid, were formed by an extract of pigeon liver. The preliminary isolation of these ribotides by paper and ion exchange chromatography and the methods of determining these compounds in reaction filtrates have been described. The specificity of the analytical methods was verified by chromatographic isolation of the ribotides. In the presence of a folic acid derivative and formate, formylglycinamide ribotide was shown to accumulate, while in its absence glycinamide ribotide was synthesized.

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ON THE MECHANISM OF SYNTHESIS OF GLYCINAMIDE RIBOTIDE AND ITS FORMYL DERIVATIVE*

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The occurrence and isolation of *N*-glycyl-5-phosphoribofuranosylamine (glycinamide ribotide) and its formyl derivative have been reported (1). This paper describes some studies on the mechanism of biosynthesis of these compounds (2).

Materials

5-Phosphoribosylpyrophosphate[1] was synthesized enzymatically and isolated by a slight modification of the procedure of Kornberg *et al.* (3, 4). PRPP used in preliminary experiments was generously supplied by Dr. A. Kornberg.[2] Glycinamide was kindly provided by Dr. J. P. Greenstein. *N*-Formylglycine was synthesized by the method of Fischer and Warburg (5). Tetrahydrofolic acid was prepared according to O'Dell *et al.* (6) as modified by Jaenicke and Greenberg.[3] 3 mg. of FAH_4 (free acid) were dissolved in 1 ml. of 0.05 M $KHCO_3$ containing 0.2 per cent ethylenediaminetetraacetate under a layer of petroleum ether and kept at 0°. The solution was prepared for each assay.

Methods

Assay Methods—The determination of glycinamide ribotide and formylglycinamide ribotide has been described in the previous paper (1). PRPP

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† DuPont Predoctoral Fellow, 1954–55. A portion of this work is included in the thesis submitted by Richard A. Peabody to the Graduate School of Western Reserve University in September, 1955, in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

[1] Abbreviations: PRPP, 5-phosphoribosylpyrophosphate; R5P, ribose-5-phosphate; ATP, adenosine triphosphate; IMP, inosine-5′-phosphate; FAH_4, tetrahydrofolic acid; CF, citrovorum factor, or calcium leucovorin (DL); PGA, 3-phosphoglyceric acid; Tris, tris(hydroxymethyl)aminomethane.

[2] The authors are also indebted to Dr. A. Kornberg and his associates for making available the procedures for the preparation of this compound.

[3] Jaenicke, L., and Greenberg, G. R., unpublished method.

was assayed at the end of the incubation by the spectrophotometric method of Lieberman *et al.* (7, 8). To precipitate the protein prior to the assay for PRPP, 0.05 ml. of 2 N $HClO_4$ was added to 0.6 ml. of the reaction mixture, and this was neutralized immediately with 0.05 ml. of 2 N $KHCO_3$. The resulting precipitate was centrifuged and an aliquot of the supernatant fluid taken for analysis as rapidly as possible. Because of the instability of PRPP in acid, all the solutions were maintained at 0°.

Enzyme Preparations—The Dowex-treated and dialyzed extract used for studies of the synthesis of formylglycinamide ribotide and glycinamide ribotide has been described (1).

The pigeon liver extract was fractionated by the following procedure. 2 gm. of acetone powder (9) were extracted at 0° for 30 minutes with 20 ml. of 0.02 M Tris buffer at pH 8.0. The mixture was centrifuged at 5000 × *g* for 30 minutes, and the supernatant solution (15 ml.) decanted into 22.5 ml. of water. 7.5 ml. of 1.0 M potassium acetate buffer[4] at pH 5.5 (measured at 1 to 10 dilution) were added slowly and the precipitate was separated by centrifugation. This fraction (acid precipitate) was washed with 10 ml. of 0.16 M potassium acetate buffer, pH 5.5, dissolved in 3.0 ml. of 0.1 M Tris buffer at pH 8.0, diluted to 15 ml. with water, and lyophilized (yield, 121 mg.). The dry powder was stable at −13° for at least 6 months.

To the supernatant solution of the acid fraction (acid supernatant fraction) approximately 0.8 ml. of 1.0 N acetic acid was added to lower the pH to 5.0. A slight precipitate was removed by centrifugation and the supernatant solution was dialyzed against 2000 ml. of 0.05 M K_2HPO_4 for 3 hours with one change of the dialysis solution. All of the above steps were performed at 2°. After dialysis the supernatant solution was lyophilized (yield, 791 mg.).

Results

Requirements for Synthesis of Formylglycinamide Ribotide—To demonstrate the cofactor requirements for this synthesis, it was necessary to treat the pigeon liver extract with Dowex 1 to remove the natural cofactors (10). The addition of boiled extract caused a 2.4-fold stimulation of formylglycinamide ribotide synthesis; citrovorum factor stimulated the synthesis 3.6-fold (Table I). These results indicated that a folic acid derivative was required for synthesis of the formylglycinamide ribotide. Homocysteine was included in those experiments, but in subsequent studies it was not required and in some instances was slightly inhibitory.

Table II presents evidence that either tetrahydrofolic acid or citrovorum factor was required for maximal synthesis. Ribose-5-phosphate, glutamine, and glycine were also necessary. In other experiments the synthesis

[4] Molarity represents the total of acetate and acetic acid.

of formylglycinamide ribotide was found to be dependent on the presence of ATP.

TABLE I

Stimulation of Formylglycinamide Ribotide Synthesis by Boiled Extract or Citrovorum Factor

Addition of cofactor	Formylglycinamide ribotide
	μmole
None	0.018
Boiled extract	0.043
CF	0.065

During a 10 minute preincubation at 38° the reaction mixture contained 20 mg. of Dowex-treated extract, 4 μmoles of DL-homocysteine, 6 μmoles of $MgCl_2$, 1.5 μmoles of ATP, 9 μmoles of PGA, 25 μmoles of K_2HPO_4, 0.5 mg. of muscle extract (11), and 0.1 ml. of boiled extract or 0.2 mg. of CF. The mixture was chilled, and the following substrates were added: 5 μmoles of C^{14}-formate with a specific activity of 20,000 c.p.m. per μmole, 5 μmoles of glutamine, 5 μmoles of glycine, 2 μmoles of R5P, and 2 μmoles of IMP; the final volume was 0.75 ml. The mixtures then were incubated for 30 minutes at 38° in air.

TABLE II

Substrate and Cofactor Requirements for Synthesis of Formylglycinamide Ribotide

Additions	Experiment 1			Experiment 2			
FAH_4	+	−	−	−	−	−	−
CF	−	+	−	+	+	+	+
R5P	+	+	+	+	−	+	+
Glutamine	+	+	+	+	+	−	+
Glycine	+	+	+	+	+	+	−
Formylglycinamide ribotide, *μmole*	0.35	0.37	0.02	0.49	0.12	0.08	0.04

The reaction mixture contained 20 mg. of Dowex-treated, dialyzed extract, 2.5 μmoles of R5P, 5 μmoles of glutamine, 5 μmoles of glycine, 6.4 μmoles of $MgCl_2$, 2.5 μmoles of C^{14}-formate, 0.2 mg. of CF or FAH_4, 1.5 μmoles of ATP, 5 μmoles of sodium phosphocreatine, and 0.05 ml. of creatine phosphokinase (aqueous rabbit muscle extract, 1:2, dialyzed 6 days *versus* water) (P. Berg, personal communication). The final volume was 0.67 ml. The mixtures were incubated for 20 minutes at 38° in air.

The accumulation of formylglycinamide ribotide at different time intervals is indicated in Fig. 1. The rate of synthesis was linear for the first 30 minutes.

Requirements for Synthesis of Glycinamide Ribotide—Glycinamide ribotide accumulated when the folic acid cofactor and formate were omitted (1).

Table III shows that the synthesis of glycinamide ribotide from glycine-1-C^{14} required ribose-5-phosphate, glutamine, and ATP.

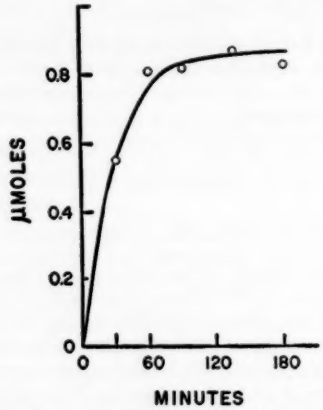

FIG. 1. Rate of accumulation of formylglycinamide ribotide. Additions to the reaction mixture were similar to those indicated in Table II, but with 1.25 μmoles of R5P and 10 μmoles of phosphocreatine.

TABLE III

Requirements for Synthesis of Glycinamide Ribotide

Omissions	Glycinamide ribotide	
	Experiment 1	Experiment 2
	μmole	*μmole*
None	0.25	0.15
R5P	0.00	0.01
Glutamine	0.01	0.02
ATP*		0.02

Additions to the reaction mixture were similar to those in Table II, but with the omission of formate and the folic acid derivative. The mixture was incubated for 30 minutes at 38° in air.

* Includes regenerating system of creatine phosphokinase (muscle extract) and phosphocreatine.

In Fig. 2 is seen the accumulation of glycinamide ribotide as a function of time. A reproducible and unexplained decrease in glycine-1-C^{14} incorporation occurred after 40 minutes. Maximal synthesis of glycinamide ribotide occurred between pH 7.0 and 8.0.

Since the incorporation of glycine-1-C^{14} was a measure of the synthesis of both the glycinamide ribotide and the formylglycinamide ribotide, the

rate-limiting reaction could be studied by a determination of the quantity of glycine-1-C^{14} incorporated in the presence and absence of the folic acid cofactor. Table IV presents the results of such an experiment. The same quantity of glycine-1-C^{14} was fixed regardless of the presence or absence of

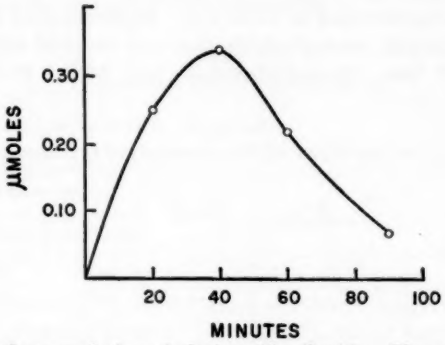

FIG. 2. Rate of accumulation of glycinamide ribotide. The additions were the same as in Table II, except that formate and the cofactor were omitted.

TABLE IV

Lack of Dependence of Glycine Incorporation on Citrovorum Factor and Formate

Special additions	Glycine-1-C^{14} incorporated
	μmole
Formate + CF	0.31
"	0.31
CF	0.27
Neither	0.30

The reaction mixture contained 2.5 μmoles of R5P, 10 μmoles of glutamine, 5 μmoles of glycine-1-C^{14}, 1.2 μmoles of ATP, 14 μmoles of PGA, 5.0 μmoles of $MgCl_2$, 0.06 mg. of muscle enzyme (11), 20 mg. of Dowex-treated extract, 5 μmoles of formate, and 0.2 mg. of CF in a final volume of 0.60 ml. The reaction mixture was incubated for 30 minutes at 38° in air.

the cofactor. Without the cofactor, no C^{14}-formate was fixed. This suggested that a reaction in the synthesis of the glycinamide ribotide was rate-limiting and that the addition of the cofactor and formate shifted a large percentage of the fixed glycine from glycinamide ribotide to formylglycinamide ribotide. Support for this concept is derived from experiments in which the products were isolated on ion exchange columns (1).

Requirement for 5-Phosphoribosylpyrophosphate—The enzyme which synthesizes PRPP (3, 4) from ribose-5-phosphate and ATP had been shown

to be present in an extract of acetone powder of pigeon liver in the protein fraction precipitated at pH 5.5 (4) (acid precipitate). The results of Experiments 1 and 2 in Table V demonstrate that the synthesis of glycinamide ribotide from ribose-5-phosphate required both the acid precipitate and the acid supernatant fraction. The requirement for ATP in these reactions was demonstrated in Table III. Experiments 2 and 3 of Table V show that the acid precipitate fraction was required only for the synthesis of PRPP from ribose-5-phosphate and ATP. In Experiment 2,

TABLE V

Requirement for PRPP and ATP in Synthesis of Glycinamide Ribotide

Additions	Acid supernatant	Acid ppt.	Glycinamide ribotide formed		
			Experiment 1	Experiment 2	Experiment 3
			μmoles	*μmole*	*μmole*
R5P + ATP	+	−	0.09	0.09	
" + "	−	+	0.07		
" + "	+	+	1.20	0.50	
PRPP	+	−		0.03	0.19
" + ATP	+	−		0.30	0.90
" *	+	−			0.03

All of the reaction mixtures contained 10 μmoles of glutamine, 5 μmoles of $MgCl_2$, 20 μmoles of Tris buffer, pH 8.0, and 5 μmoles of glycine-1-C^{14}. The additions indicated were 2.5 μmoles of R5P in Experiment 1 and 5 μmoles of R5P in Experiment 2, 1.2 μmoles of ATP with a regenerating system of 14 μmoles of PGA and 0.6 mg. of muscle extract (11), 1.1 μmoles of PRPP, 20 mg. of the lyophilized acid supernatant fraction, and 1.2 mg. of the acid precipitate fraction. The final volume in Experiment 1 was 0.85 ml. and 1.1 ml. in Experiments 2 and 3. The reaction mixture was incubated for 30 minutes at 38° in air.

* PRPP was preincubated for 10 minutes at 38° with 0.1 ml. of apyrase (12) to destroy ATP. This enzyme does not hydrolyze PRPP. The indicated additions then were made prior to the 30 minute incubation.

PRPP substituted for this fraction and ribose-5-phosphate, but ATP was still required. In Experiment 3 similar results were obtained with a different enzyme preparation. In this experiment PRPP was preincubated with apyrase (12) to remove a small amount of ATP which contaminated the preparation. The apyrase preparation did not degrade the PRPP.

Reaction of PRPP with Glutamine—PRPP was found to react with glutamine and not with glycine (Table VI) in the presence of the acid supernatant enzyme. The high blank may be due to incomplete removal of glutamine from the enzyme preparation because of the short dialysis period. While in these experiments ATP was added, Table VII shows that the reaction of PRPP with glutamine did not require ATP.

Evidence against the participation of glycinamide in this synthesis was obtained by the finding that a pool of unlabeled glycinamide did not alter the incorporation of glycine-1-C^{14} into glycinamide ribotide. By a similar type of experiment, formylglycine has been shown not to be an intermediate in the synthesis of formylglycinamide ribotide.

TABLE VI

Reaction of PRPP with Glutamine

Amino acid additions	PRPP utilized
	μmole
None	0.06
Glutamine	0.11
Glycine	0.06
Glutamine + glycine	0.11

The reaction mixture contained 0.26 μmole of PRPP, 1.2 μmoles of ATP, 14 μmoles of PGA, 1.2 mg. of muscle enzyme (11), 5.0 μmoles of $MgCl_2$, 20 μmoles of Tris buffer, pH 8.0, 10 mg. of lyophilized acid supernatant fraction, 5 μmoles of glutamine, and 2.5 μmoles of glycine in a final volume of 0.6 ml. The mixture was incubated for 15 minutes at 38° in air.

TABLE VII

Reaction of PRPP with Glutamine

Additions	PRPP utilized
	μmole
None	0.02
Glutamine	0.17
" + ATP*	0.17

The reaction mixture contained 0.43 μmole of PRPP, 5.0 μmoles of $MgCl_2$, 20 μmoles of Tris buffer, pH 8.0, 10 mg. of lyophilized supernatant fraction, 5 μmoles of glutamine, and 1.0 μmole of ATP. The final volume was 0.5 ml., and the mixture was incubated for 20 minutes at 38°.

* When ATP was added, 7 μmoles of PGA and 1.2 mg. of the muscle enzyme (11) were also included as an ATP-regenerating system.

DISCUSSION

In recent years it has been suggested that intermediate compounds in the synthesis of the purine ring are ribose phosphate derivatives (13–15). Such compounds as 5-amino-4-imidazolecarboxamide ribotide (10, 16, 17) and a compound having the properties of 5-aminoimidazole riboside or ribotide (18, 19) have been isolated and shown to be intermediates in the synthesis of purine nucleotide derivatives. The occurrence of two new ali-

phatic precursors, glycinamide ribotide and its formyl derivative, strengthens the concept that ribotide compounds are intermediates in the synthesis of inosine-5′-phosphate. In the present study evidence is presented that even the initial steps in the biosynthetic scheme involve ribose-5-phosphate derivatives. Kornberg, Lieberman, and Simms have shown a reaction between ribose-5-phosphate and ATP to yield 5-phosphoribosylpyrophosphate (3, 4). In experiments reported in this paper, the involvement of PRPP in the synthesis of glycinamide ribotide has been presented. In addition it has been demonstrated that ATP is required not only for the synthesis of PRPP, but also for a later step in the sequence of reactions. It has been found recently that ATP is required for the reaction of glycine with 5-phosphoribosylamine to form glycinamide ribotide (20). It should be pointed out that Hartman, Levenberg, and Buchanan (21) have shown independently the participation of PRPP, and the ATP requirement in the synthesis of glycinamide ribotide.

Although several compounds such as glutamine, glycine, and ribose derivatives (13, 22, 23) have been known to stimulate the synthesis of hypoxanthine, the exact sources of the carbon and nitrogen atoms of the purine ring were elucidated with isotopically labeled precursors (24–28). Sonne, Lin, and Buchanan (27) and Lagerkvist (28) have reported that N_3 and N_9 of the purine ring are derived from the amide nitrogen of glutamine. It is reasonable to conclude that the amide nitrogen of glycinamide ribotide becomes N_9 of the purine ring.

It is clear from these studies that the formylation of glycinamide ribotide requires a folic acid derivative. The introduction of this carbon, which becomes position 8 in the purine (21), appears to occur by mechanisms which are analogous to those involved in the reaction of formate and 5-amino-4-imidazolecarboxamide ribotide to form inosine-5′-phosphate. Recently the conversion of formylglycinamide ribotide to 5-aminoimidazole ribotide and then to the 5-amino-4-imidazolecarboxamide ribotide has been reported (19).

SUMMARY

The mechanism of synthesis of glycinamide ribotide and formylglycinamide ribotide in extracts of pigeon liver acetone powder has been studied. For synthesis of both ribotides, ribose-5-phosphate, ATP, glutamine, and glycine were required. In addition the synthesis of formylglycinamide ribotide required formate and a folic acid derivative. By fractionation of the enzyme system, 5-phosphoribosylpyrophosphate (PRPP) was shown to substitute for ribose-5-phosphate, but ATP was still required. A reaction between PRPP and glutamine has been demonstrated.

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SOME BIOCHEMICAL CHANGES ACCOMPANYING PENICILLIN INHIBITION OF SARCINA LUTEA*

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Penicillin has been reported to cause alterations in the bacterial metabolism of amino acids (1), peptides (2), nucleotides (3), and a unique series of molecules containing both nucleotides and peptides as well as hexosamine (4). Therefore, as a preliminary attempt to detect the earliest consequences of penicillin action, a time-related survey of changes in these types of cellular components was indicated. This report summarizes the results of such a survey on a penicillin-sensitive, rough variant of *Sarcina lutea*.

EXPERIMENTAL

Cell Growth and Harvest—Stock cultures of a rough variant of *Sarcina lutea* (ATCC 9341) described previously (5) were transferred twice weekly on Difco AC agar slants and incubated at room temperature. Inhibition studies were made on cells grown in synthetic broth of the following composition (per liter and adjusted to pH 7.0): casamino acids (Difco) 8.0 gm., dextrose 5.0 gm., potassium acetate 3.0 gm., ammonium sulfate 5.0 gm., guanine 5.0 mg., niacin 200.0 γ, thiamine 200.0 γ, Salt Solutions A and B (6) 2.5 ml. each, Tween 80 (Atlas Powder Company) 0.1 ml. The broth was sterilized by autoclaving in 2 liter diphtheria toxin bottles. Cells from a 24 to 48 hour slant were transferred by an inoculating loop to a bottle containing 0.5 to 1.0 liter of sterile broth, which was incubated at 25° for 24 hours on a reciprocal shaker operating at 180 strokes per minute. By sterile technique 0.25 liter of this broth inoculum was added to each of two or three bottles containing 0.75 liter of sterile medium, and these cultures were then incubated with shaking. Throughout the test period, growth was determined turbidimetrically on aliquots from each bottle. Optical density readings were made at 540 mμ in calibrated 15 × 125 mm. rimless tubes on a Coleman junior spectrophotometer. After the turbidi-

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ties had reached 0.35 to 0.40 optical density unit (2 to 3 hours following inoculation), 1 ml. or less of a concentrated solution of potassium penicillin G (Bristol Laboratories, Inc.) was added to one of the bottles, so that the concentration of the antibiotic in this culture was 1 unit per ml.

In initial survey experiments, three test bottles were used: one was removed from the shaker at the same time that penicillin was added to a second bottle; the third bottle served as a control over the 3 hour period of exposure to the antibiotic. Cells from the total contents of the three bottles were harvested by centrifugation at about 1000 × *g* for 20 minutes on an International centrifuge.

For time study experiments, only two bottles were used: one to which penicillin was added and one which served as a control. By use of a volumetric pipette, 50 ml. aliquots were withdrawn from each bottle at various intervals, turbidities read, and cells harvested by centrifugation on a Servall low speed centrifuge at about 3000 × *g*.

In either case, cells were washed once with 20 ml. of distilled water, centrifuged on the Servall centrifuge at 3000 × *g*, and transferred quantitatively to small VirTis flasks for lyophilization. The dried cells were carefully recovered from the flasks and weighed.

Extraction of Acid-Soluble Components—The weighed cells (not exceeding 100 mg.) were extracted twice with 10 ml. of 5 per cent trichloroacetic acid (TCA) at 5° on a wrist action shaker for a total period of 6 hours. Cell residues were removed by centrifugation on the Servall centrifuge at 3000 × *g* and TCA was removed from the supernatant fluids by extraction with ether.

Hydrolysis of Cell Residues—The residue from TCA extraction of 10 mg. of dry cells was hydrolyzed with 4 ml. of 6 N HCl for 24 hours.

Methods of Analysis—Absorption at 260 mμ was read on a Beckman model DU spectrophotometer. Total nitrogen on cell residues was determined by the micro-Kjeldahl method. Amino nitrogen was estimated by using the naphthoquinone sulfonate reagent of Russell (7). Peptide nitrogen in the TCA extracts was calculated from amino nitrogen difference before and after hydrolysis with 6 N H_2SO_4 for 18 hours. Aliquots of cell residue hydrolysates were evaporated to dryness *in vacuo* prior to amino nitrogen analysis. Hexosamine was determined by the Boas modification (8) of the Elson and Morgan procedure. The method of Fiske and Subbarow (9) was used for phosphate analysis. Hydrolysis for 12 minutes in 1 N HCl at 92° was used in the determination of "pyro" phosphate. Proline was estimated by using the ninhydrin reagent of Schweet (10). It was found advantageous to evaporate the sample to dryness *in vacuo* before addition of this reagent.

Paper Chromatography—The two-dimensional system reported by Red-

field (11) was used. TCA extracts were concentrated about 20-fold and 0.01 ml. was applied to the corner of each paper.

TABLE I

Variation in Turbidity, Cell Weight, and Nitrogen Content of Control (A) and Penicillin-Treated (B) Cultures of S. lutea with Time

	Initial		½ hr.		1 hr.		2 hrs.		4 hrs.	
	A	B	A	B	A	B	A	B	A	B
Turbidity, *optical density* × *100 at 540 mμ*	32	32	38	37	42	38	51	43	56	43
Weight, *mg. dry cells per 50 ml. aliquot of culture*	28	30	35	35	42	39	54	44	64	48
Turbidity / Weight	1.14	1.07	1.08	1.06	1.00	0.98	0.94	0.98	0.88	0.90
Nitrogen content of TCA-extracted residue, *mg. per 10 mg. dry cells*	0.78	0.78	0.77	0.77	0.73	0.81	0.75	0.77	0.79	0.80

TABLE II

Survey of Effect of Penicillin on Acid-Soluble Components of S. lutea

The figures are in micrograms per 10 mg. of dry cells.

	Control (0 hr., 0.392 O. D. unit*)	Penicillin-treated, 1 i.u. per ml. (3 hrs., 0.432 O. D. unit*)	Control (3 hrs., 0.327 O. D. unit*)
Phosphate			
Inorganic	36	34	35
"Pyro"	12	11	11
Total	65	64	69
Nitrogen			
Free amino	70	54	84
Peptide†	13	15	15
Hexosamine	8	11	10
Total	116	104	157

* Absorption at 260 mμ; O. D. = optical density. Each ml. of extract contains the TCA-extractable material in 1 mg. of dry cells.

† From amino nitrogen difference before and after hydrolysis with 6 N H_2SO_4 for 18 hours.

Results

Effect of Penicillin on Culture Turbidity, Cell Weight, and Nitrogen Content—Table I summarizes the data for intervals within a 4 hour period.

From a comparison of culture turbidities at initial and final time of exposure to penicillin, a growth inhibition of about 55 per cent may be calculated for the treated cells. Although the turbidity to weight ratio is not constant for all times, no difference was noted between treated and un-

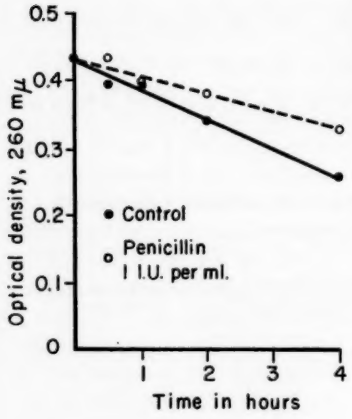

FIG. 1

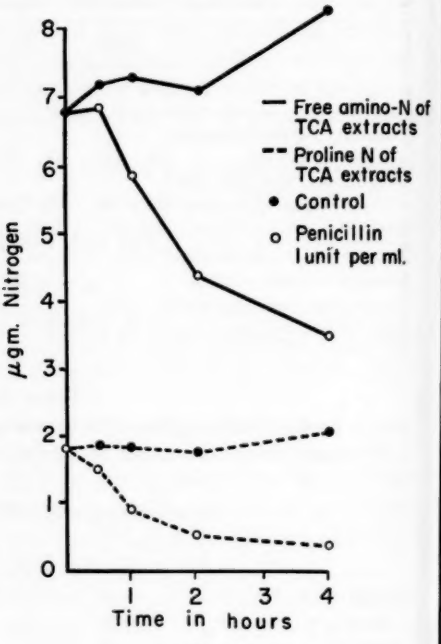

FIG. 2

FIG. 1. The effect of penicillin on absorption of TCA extracts of *S. lutea* at 260 mμ. Each ml. of extract contains the acid-soluble material in 1 mg. dry weight of cells.

FIG. 2. The effect of penicillin on free amino nitrogen and proline nitrogen of TCA extracts of *S. lutea*. All values are per mg. dry weight of cells.

treated cultures in this respect. The nitrogen content of TCA-extracted cell residues remained essentially constant relative to whole cell dry weight.

Survey of Effect of Penicillin on Acid-Extractable Components—A quantitative comparison of various types of TCA-extractable cellular constituents is provided in Table II. At the end of 3 hours, extracts of penicillin-treated cells showed a slight increase in absorption at 260 mμ compared to controls. However, phosphate values remained constant. The total nitrogen content of treated cell extracts dropped considerably, owing in large measure

to a decrease in free amino nitrogen. Peptide and hexosamine values remained comparable to those of the controls. Paper chromatography of the extracts revealed that proline had almost completely disappeared while other amino acids had undergone only slight changes.

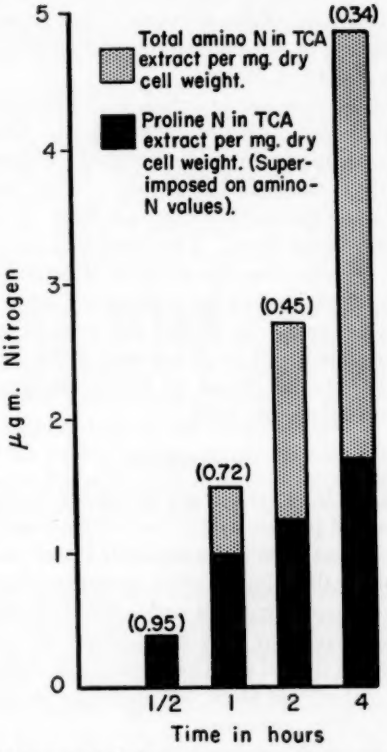

FIG. 3. Proline nitrogen and amino nitrogen lost from penicillin-treated cells. The numbers in parentheses give the ratio of proline nitrogen to total amino nitrogen lost.

Time-Related Effect of Penicillin on Certain Cellular Constituents—Fig. 1 shows that TCA extracts of penicillin-treated cells lose material absorbing at 260 mμ less rapidly than control extracts. Although these values in different experiments are somewhat erratic, especially at the ½ and 1 hour intervals, no significant difference has ever been noted during early exposure to the antibiotic.

Hydrolyzed cell residues from TCA extraction were analyzed for proline

and free amino nitrogen. At all times (0, ½, 1, 2, and 4 hours) and in the presence or absence of penicillin, these values remained quite constant at 2.4 γ of proline nitrogen and at 43 to 46 γ of amino nitrogen per mg. dry cell weight. Since the molar extinction coefficient of secondary amines (proline) is only one-half that of primary amines with the naphthoquinone sulfonate reagent, all total amino nitrogen values reported have been corrected by the addition of one-half of the proline nitrogen weight as determined with the ninhydrin reagent.

By contrast to other constituents tested, TCA-extractable amino nitrogen dropped sharply in penicillin-treated cells (Fig. 2). Quantitative tests for proline indicated that this amino acid accounted for almost all of the amino nitrogen losses early in the period of exposure to the antibiotic. In Fig. 3 the difference in acid-extractable amino nitrogen of control and treated cells is graphed at various times. Corresponding proline nitrogen values are superimposed. ½ hour after the addition of penicillin, proline is noted to make up about 95 per cent of the difference. After 4 hours this value has dropped to 34 per cent. In normal cell extracts, at all the times recorded, proline accounts for 24 to 26 per cent of the total amino nitrogen. It appears that the ratio of proline to amino nitrogen lost from treated cells might be approaching this limit.

DISCUSSION

If cultures of the *S. lutea* variant are inoculated by drop suspension and incubated as described previously (5) but without shaking, growth is inhibited about 50 per cent in 24 hours by 0.002 i.u. of penicillin per ml. It may appear, therefore, that the penicillin concentration used in the present experiments is exceptionally high for such a sensitive organism. Some explanation for this discrepancy may be obtained by a comparison of cell numbers present at the time of addition of penicillin in the two experiments, and it can be calculated that about 10^5 molecules of penicillin are present in the medium per cell in both cases.

In experiments in which synthetic amino acids replaced the casein acid hydrolysate, omission of proline from the mixture has been found to prolong the lag phase of cell growth by about 6 hours compared to cells grown in this medium plus proline (0.5 mg. per ml.). In view of this fact one might conclude that the penicillin sensitivity of the organism relates to this partial requirement for proline. If penicillin were assumed to act by blocking the assimilation of proline, the addition of the antibiotic during periods of rapid multiplication of the bacteria would be expected to damage protein synthesis extensively before the cell could adapt to the *de novo* synthesis of the amino acid. A situation similar to the "growth imbalance" proposed by Cohen and Barner (12) might exist. However, cells adapted to

the lack of proline were found not only to grow at precisely the same rate as the unadapted controls but also to respond to penicillin added during the logarithmic phase to exactly the same rate and extent.

In experiments similar to ours, Gale and Taylor (1) demonstrated a marked decrease in the acid-extractable glutamic acid of staphylococci when penicillin was added to cells in the logarithmic phase. Gale later noted (13) the same effect using the uncoupling reagent, dinitrophenol. The uptake of most of the other amino acids was inhibited only slightly by comparison to glutamate (14). However, dinitrophenol diminished proline assimilation by a factor of almost 2 compared to glutamate. This evidence suggests that these two amino acids are highly dependent on an energy-requiring "pump" for their assimilation. The mechanism of this pump remains obscure, but it is possible that the uridine pyrophosphate compounds which Park (4) found to accumulate in penicillin-treated staphylococci are involved.

It seems unlikely that penicillin acts directly on a proline-specific enzyme system, although the similarity between the pyrollidine and thiazolidine rings makes competitive inhibition an inviting hypothesis. However, attempts to reverse penicillin with proline and a proline peptide (glycylproline) failed. Interpretation of the data obtained so far would affirm Gale's hypothesis that penicillin acts on amino acid assimilation mechanisms. Proline, an amino acid most dependent on these mechanisms, appears to be most drastically affected in this instance.

SUMMARY

Growth of aerated cultures of a rough variant of *Sarcina lutea* was inhibited about 50 per cent when the cells were exposed to penicillin (1 i.u. per ml.) for 3 to 4 hours. Analysis of the cells from aliquots of the culture taken at various time intervals revealed that a rapid drop in acid-extractable amino nitrogen preceded all other alterations measured. Proline accounted for almost all of this loss within 30 minutes following addition of penicillin. By the end of the 4 hour period, alterations in other nitrogenous constituents were noted and proline made up only about 35 per cent of the acid-extractable amino nitrogen difference.

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UTILIZATION OF D-GLUCOSE-1-C^{14} BY TORULA UTILIS YEAST. II

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In a previous publication (1), data were reported concerning D-mannose and D-ribose isolated from *Torula utilis* yeast which had been grown on D-glucose-1-C^{14} as the sole source of carbon. It was found that water extraction of the lyophilized yeast yielded a mannan that was completely devoid of radioactivity. Further, the D-ribose obtained from the purine ribosides of the nucleic acid fraction showed a low specific radioactivity, suggesting that the major pathway for its production was via the oxidative decarboxylation and isomerization of D-gluconic acid 6-phosphate.

The present report is concerned with the separation from the same yeast sample, by extraction with 6 per cent sodium hydroxide solution, of a second, radioactive mannan composed of polymerized D-mannose-1-C^{14}. Further alkaline extraction, with 30 per cent potassium hydroxide solution, yielded a polysaccharide fraction containing both radioactive D-glucose and D-mannose.

Methods

Isolation of Mannan—The sample used in the present work was the residue of *T. utilis* yeast described previously (1), which had been extracted in the cold with ethanol-ether (3:1), water (pH 7), and 2.2 per cent sodium hydroxide solution. This residue (32.7 gm.) was treated with 200 ml. of 6 per cent sodium hydroxide solution at 4° under continuous agitation for 24 hours. The suspension was centrifuged at 10,000 r.p.m. in a Servall superspeed angle centrifuge to remove the insoluble residue. The latter was extracted immediately for "glycogen" as described in a later section. The brown supernatant liquor (150 ml.) was neutralized with 19 ml. of concentrated hydrochloric acid, whereupon a buff-colored material precipitated. This was removed by centrifugation and an equal volume of 95 per cent ethanol was added to the supernatant solution. The resulting white precipitate was washed with 95 per cent ethanol, then with ether, and dried *in vacuo*. The yield was 2.2 gm. of a fine white powder. The carbohydrate content of this material, by the Somogyi-Nelson method, was 82.5 per cent calculated as glucose, corresponding to a polysaccharide con-

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tent of 74.2 per cent. The protein content, calculated from a total nitrogen value of 3.8 per cent, was 23.8 per cent.

Paper chromatography of an acid hydrolysate revealed the presence of both glucose and mannose, the latter being present in greatly preponderant amount by visual estimation.

Isolation of D-*Mannose*—A sample of the above polysaccharide-protein material (1.5 gm.) was hydrolyzed and treated with phenylhydrazine in the manner described previously (1) to yield 1.1 gm. of D-mannose phenylhydrazone, m.p. 198–199°. Crystalline D-mannose was obtained readily by crystallization from ethanol after cleavage of the hydrazone (1) in a yield of 340 mg., m.p. 130°, $[\alpha]_D^{20}$ 14.2° (final in water). The sugar produced a single spot, corresponding to and superimposable on that from known D-mannose, upon one-dimensional paper chromatography in water-saturated phenol and in water-saturated lutidine.

TABLE I

Specific Radioactivities of D-*Mannose and Its Derivatives from Mannan*

Component	Mg. per sq. cm.	C.p.m. per μmole
D-Mannose	0.100	54
" phenylhydrazone	0.100	50
Benzimidazole	0.087	48

Radioassay of D-*Mannose and Derivatives*—Carbon-1 of the D-mannose was isolated from the other carbons of the sugar structure through synthesis of D-mannobenzimidazole (2), followed by oxidation and decarboxylation to benzimidazole (3). The latter, as well as the D-mannose and D-mannose phenylhydrazone, was radioassayed as thin samples (about 0.1 mg. per sq. cm.) in a gas flow counter with an autoscaler attachment. Since all the samples were mounted under nearly identical conditions, no corrections were made for self-absorption. Table I lists the specific radioactivities and the thickness of the mounts for the samples from mannan.

Isolation of "Glycogen"—The insoluble residue from the 6 per cent sodium hydroxide extraction was suspended in 75 ml. of 30 per cent potassium hydroxide solution (weight per volume) and heated in a boiling water bath for 3 hours with occasional agitation. The suspension was then cooled and filtered through glass wool under slight suction. The filtrate was diluted with water to a volume of 300 ml., and 360 ml. of 95 per cent ethanol were added (final ethanol concentration, 52 per cent). A dense, light colored precipitate in a brown solution resulted. After storage at 4° for 24 hours, the precipitate was removed by centrifugation, resuspended in 100 ml. of 30 per cent potassium hydroxide solution, and the precipitation with

ethanol repeated. After washing with 52 per cent ethanol the colorless precipitate was extracted by stirring with 160 ml. of water and the insoluble residue was removed by centrifugation. Ethanol was added to the aqueous extract to a final alcoholic concentration of 52 per cent, followed by dilute hydrochloric acid to the Congo red end-point, and the precipitate was collected by centrifugation. This alcoholic hydrochloric acid precipitation was repeated four times. The sample then was precipitated twice from aqueous solution with four volumes of glacial acetic acid. Finally, the product was washed two times each with 52 per cent ethanol, with 95 per cent ethanol, with absolute ethanol, and with acetone. After drying *in vacuo*, the resulting "glycogen" was a fine white powder weighing 580 mg.

Analysis of the "glycogen" by the Somogyi-Nelson method showed a carbohydrate content of 102 per cent calculated as glucose, or 92 per cent as polysaccharide. There was no detectable nitrogen in the product. The optical rotation, $[\alpha]_D^{20}$ 40.4° in water, was lower than expected for normal glycogen, and paper chromatography of an acid hydrolysate revealed both mannose and glucose in approximately equal amounts by visual estimation. Furthermore, the relative concentrations of the two sugars were not significantly altered during the above purification procedures.

TABLE II

Specific Radioactivities of Derivatives of D-*Mannose and* D-*Glucose from "Glycogen"*

Component	Mg. per sq. cm.	C.p.m. per μmole
Nutrient D-glucose	0.106	75
D-Mannose phenylhydrazone	0.100	52
D-Glucosazone from D-mannose	0.095	53
" " D-glucose	0.101	51

D-Glucose and D-Mannose from "Glycogen"—D-Mannose was precipitated from an aliquot of the acid-hydrolyzed "glycogen" as the phenylhydrazone. Both the D-glucose and D-mannose phenylhydrazones then were converted separately to D-glucosazone (4). There were obtained from the aliquot 10.6 mg. of the osazone from D-glucose, m.p. 206–207°, and 9.0 mg. of the osazone from D-mannose, m.p. 205–206°, indicating nearly equal concentrations of D-glucose and D-mannose in the "glycogen" fraction.

The specific radioactivities of the osazones from each sugar and of the original D-glucose used in the yeast growth medium are shown in Table II.

RESULTS AND DISCUSSION

It is apparent from Table I that essentially all of the radioactivity present in the D-mannose of this mannan is located in carbon-1. It thus may

be concluded that the D-mannose was produced directly from D-glucose without rearrangement of the carbon chain. This result is similar to that obtained by Gilvarg (4) for a mannan from *Saccharomyces cerevisiae* yeast grown on D-glucose-1-C^{14} in the presence of acetate.

As was suggested in the previous publication (1), there are present in our *T. utilis* yeast preparation two D-mannose polysaccharides which were synthesized in different ways and which differ in their mode of retention in the yeast cell.

From Table II it is noted that the D-glucose obtained from the yeast "glycogen" showed 51 c.p.m. per μmole, whereas the original D-glucose used in the yeast growth medium showed 75 c.p.m. per μmole. This decrease in specific radioactivity indicates the resynthesis of D-glucose of low, or no, radioactivity. Recombination of triose phosphate produced by the glycolytic pathway would not be expected to cause this reduction in radioactivity, but rather to cause redistribution of the label with unchanged total isotope content. Thus, if the triose recombination process were of major importance, the relative amount of label in carbon-1 of the D-glucose and D-mannose to that elsewhere in the sugar chain should be significantly reduced. Since essentially all of the radioactivity in the D-mannose is located in carbon-1, it is concluded that triose recombination does not occur to any important extent under the conditions employed.

At least two explanations could account for the observed dilution of radioactivity. In the previous publication (1) evidence was advanced that the oxidative shunt pathway was involved in the production of the D-ribose of the yeast nucleic acid. The reversibility of this pathway, which has been demonstrated by Horecker and Smyrniotis (5), could result in D-glucose of lowered isotope content through dilution of carbon dioxide from carbon-1 with that from the non-radioactive carbons of the D-glucose-1-C^{14}. Moreover, the non-radioactive D-mannose utilized in forming the water-extractable mannan (1) may have been partially reconverted to D-glucose, thus diluting the radioactivity of the D-glucose pool.

The observation that the D-mannose comprising the radioactive mannan and that from the "glycogen" fraction had the same radioactivity as the D-glucose from the latter suggests that D-mannose was produced by isomerization and incorporated into the polysaccharide concurrently with D-glucose.

The inability to lower the D-mannose content of the "glycogen" fraction by several reprecipitations with acid ethanol or acetic acid suggests the existence of a glucomannan in this yeast preparation.

SUMMARY

In addition to the previously reported non-radioactive mannan, a second, radioactive mannan has been isolated from *Torula utilis* yeast grown on

D-glucose-1-C^{14} as the sole source of carbon. Essentially all of the radioactivity in this second mannan is located in carbon-1 of the D-mannose.

The D-mannose from this second mannan and the D-glucose and D-mannose from the "glycogen" fraction possessed identical specific radioactivities. It is suggested that the D-mannose was produced by isomerization from D-glucose and condensed into polysaccharide concurrently with D-glucose.

The relative concentrations of D-mannose and D-glucose in the "glycogen" fraction were unchanged by several fractional precipitations. The presence of a glucomannan is suggested.

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A BACTERIAL RIBOFLAVIN HYDROLASE*

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Riboflavin is known to be decomposed by specific bacteria. Foster (1, 2) showed that the ribityl portion of riboflavin was oxidized by living cells of *Pseudomonas riboflavina*, leaving the heterocyclic ring, 7,8-dimethylalloxazine (lumichrome), intact. Later, Hou (3) found that lumichrome accumulated as riboflavin disappeared in a culture of tubercle bacilli to which the vitamin had been added. The present paper deals with the demonstration and characteristics of a bacterial cell-free enzyme which hydrolyzes riboflavin to lumichrome and ribitol. The name riboflavin hydrolase is proposed for this new enzyme.

Methods and Materials

Isolation and Cultivation of Riboflavin-Decomposing Bacterium—The bacterium used in this experiment was freshly isolated as described earlier (1) from soils enriched with riboflavin. When the pure culture was streaked and incubated for a few days at 30° on a solid medium containing, per liter of distilled water, 500 mg. of $MgSO_4 \cdot 7H_2O$, 500 mg. of KH_2PO_4, 2 gm. of yeast extract (Difco), 200 mg. of riboflavin, and 20 gm. of agar (Difco), it decolorized the medium in the proximity of the colonies. Also, characteristic bright yellow crystals of lumichrome accumulated in the colonies.

The concentration of riboflavin in the medium was found to be one of the limiting factors in the formation of the riboflavin-decomposing enzymes in the cell. Cells harvested from a liquid medium containing less than 30 mg. of riboflavin per liter possessed no or only slight riboflavin hydrolase activity. The presence of 1 per cent glucose in the above medium resulted in more abundant growth of the organism, but in negligible riboflavin-decomposing activity. The best liquid medium for obtaining an active cell-free preparation was the yeast extract-salts medium mentioned above containing 100 mg. of riboflavin per liter. Morphologically and culturally the bacterium is identical with *P. riboflavina* (1).

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Preparation of Cell-Free Enzymes—The bacteria were cultured with mechanical shaking for 30 hours at 30° in 3 liter wide bottom flasks each containing 1 liter of the liquid medium. The bacteria were harvested with a Sharples steam-driven centrifuge and washed several times with water to remove a yellow mucoid substance which coated the cells. Cells thus obtained from 3 liters of medium were suspended in 22 ml. of 0.01 M Tris(tris (hydroxymethyl)aminomethane) buffer (pH 7.0), and one-half of this suspension was sonically vibrated for 1 hour at 4° in a Raytheon 10 kc. magnetostrictor oscillator. The other half of the suspension was stored at 4° for later comparison with the activity of the cell débris. The sonic extract was centrifuged at 880 × *g* in the cold. The clear supernatant fluid was separated and the débris was washed twice with 0.01 M Tris buffer. The débris was finally resuspended in 11 ml. of the same buffer.

Analytical Methods—Riboflavin used throughout these experiments was purchased from the Matheson Company, Inc., and from Merck and Company, Inc. The authentic lumichrome was that previously prepared microbiologically (1); it was recrystallized from a pyridine-ethanol mixture (4). C.p. adonitol (synonym of ribitol) (Pfanstiehl) was used as authentic ribitol.

The absorption spectrum of lumichrome was obtained with a Beckman model DU spectrophotometer. Riboflavin was determined quantitatively by means of a Pfaltz and Bauer fluorometer with filter Nos. 3384 and 3389 placed between the cuvette and the photocell. Within the concentration range employed (0.5 to 3.3 × 10^{-7} M) fluorescence was directly proportional to the amount of riboflavin. All fluorometric analyses were performed on samples containing 1 per cent acetic acid. A small correction was employed to account for the fluorescence of lumichrome formed in the reaction mixture as riboflavin was decomposed. The fluorescence of lumichrome was found to be 0.148 that of an equimolar concentration of riboflavin. Although an error of this magnitude is too small to have a significant effect on the results or their interpretation, the concentration of riboflavin, in the presence of lumichrome produced from it in any particular reaction mixture, was computed from a nomograph constructed for that purpose.

EXPERIMENTAL

Fractionation of Enzyme Activity—Riboflavin hydrolase activities of intact cells, of sonic extract supernatant fluid, and of sonic extract débris (containing some intact cells) were compared. The activity of the supernatant fluid was relatively negligible in relation to that of the débris and of the intact cells. From this experiment it was concluded that the cell débris, though still containing some intact cells, exhibited considerably stronger enzyme activity than a suspension of the same number of intact cells. Therefore an attempt was made to obtain a preparation of active débris devoid of whole cells.

From 12 liters of culture 19.7 gm. of washed wet cells were obtained and sonically vibrated in 60 ml. of Tris buffer. The sonic extract was centrifuged for 30 minutes at 880 × *g*. This removed essentially all the cells and large débris particles. The turbid supernatant liquid was recentrifuged in a Spinco centrifuge for 1 hour at 12,200 × *g*, and the clear supernatant fluid (62 ml.) was collected and stored at 4°. The precipitate formed two layers, a trace of a bottom layer of opaque, yellow, undisintegrated cells and a fairly thick upper layer consisting of small particles in the form of a semitransparent, light brownish gel. The latter was separated mechanically from the former and washed a few times with Tris buffer by high speed centrifugation (12,200 × *g*). The above procedures were carried out in the cold. As demonstrated in Table I, the supernatant fluid again ex-

TABLE I

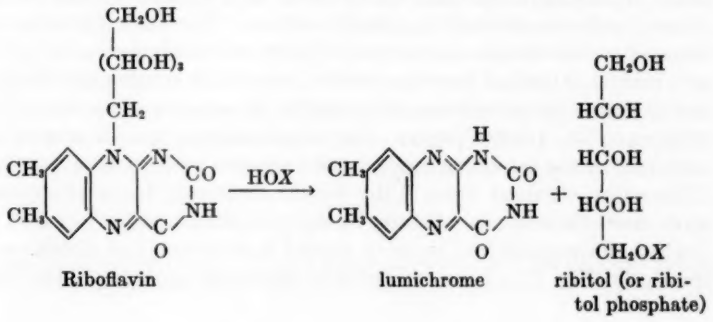
Riboflavin-Decomposing Activities of Two Fractions of Bacterial Sonic Extract

The reaction system consisted of 0.5 ml. of 1.82×10^{-5} M riboflavin, 0.5 ml. of 0.02 M Tris buffer (pH 7.0), and 0.5 ml. of supernatant or 0.2 ml. of particle suspension plus 0.3 ml. of water. These were shaken in a water bath at 32° for 2 hours. The reaction was stopped by adding 3.5 ml. of alcohol containing 5 per cent acetic acid. The solutions were clarified by centrifugation. Fluorescence of the supernatant solutions was measured after appropriate dilution. Initial riboflavin concentration was 1.82×10^{-5} M.

Enzyme preparation	Final riboflavin concentration	Per cent decomposition
	M	
Particles	7.5×10^{-6}	61.3
Supernatant	1.70×10^{-5}	9.0

hibited only slight activity, while the cell-free suspension of small particles showed a definite enzyme activity.

Identification of Reaction Products—Conversion of riboflavin to lumichrome could reasonably be considered as a hydrolysis or a phosphorolysis



Riboflavin lumichrome ribitol (or ribitol phosphate)

reaction as shown in the accompanying reaction, where X represents H or PO_3H_2. The reaction products were analyzed along this line in the following experiments.

Lumichrome—Small particle fraction (1.3 ml.), 2 mM riboflavin (1.0 ml.), and 0.02 M Tris buffer (1.7 ml.) were mixed and incubated at 32° for 4 hours. The reaction was stopped by adding 21 ml. of ethanol containing 1 ml. of acetic acid. The precipitate formed was centrifuged and washed twice with 2 ml. of ethanol. The clear supernatant fluid and the washings were

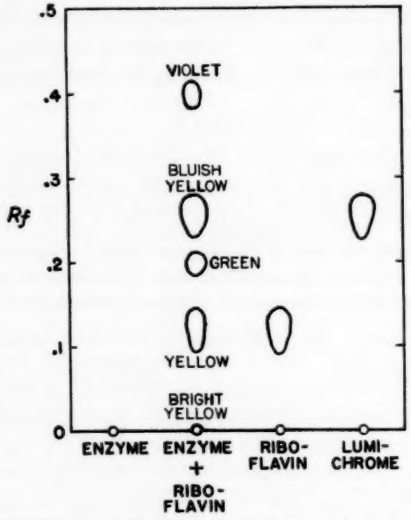


FIG. 1. Paper chromatogram of enzyme, riboflavin, and lumichrome. Conditions described in the text. Colors of spots refer to fluorescence in ultraviolet light.

combined and then evaporated *in vacuo*. The yellow syrupy residue thus obtained was again extracted with ethanol on a steam bath and the clear extract was concentrated to a small volume. The same procedure was followed for the enzyme preparation without added riboflavin, this serving as a control. Both of the concentrates, along with solutions of riboflavin and of authentic lumichrome, were applied as separate spots to a sheet of Whatman No. 1 filter paper. The chromatogram was developed with methanol by the ascending method and examined with an ultraviolet lamp (Mineralite obtained from Ultra-Violet Products, Inc.). Fluorescent spots from the reaction mixtures at R_f 0.26 (bluish yellow) and at 0.10 (yellow) corresponded to those of control lumichrome and riboflavin, respectively (Fig. 1). Three unidentified fluorescent spots were observed at

R_F 0.00, 0.19, and 0.40, these being, respectively, bright yellow, green, and violet[1] in fluorescence and very weak in intensity. The strong fluorescent area corresponding to the control lumichrome spot was cut out and eluted with 0.1 N Na_2CO_3 at room temperature, neutralized with dilute acetic acid, and evaporated to dryness *in vacuo*. The residue was extracted with hot ethanol and after evaporation of the ethanol the residue was taken up in 0.1 N Na_2CO_3. The absorption spectrum of this yellow solution showed maxima at 256, 348, and 396 mμ. These corresponded with those of authentic lumichrome (258, 350, and 395 mμ).

Tests for Organic Phosphate Compounds—Riboflavin (2 mM, 0.5 ml.) was decomposed by the enzyme preparation (1.5 ml.) in the presence of 0.02 M phosphate buffer at pH 7.0 (0.2 ml.). The reaction was stopped after incubation for 2 hours at 32° by the addition of 10 ml. of ethanol. After removal of the precipitate the supernatant liquid was evaporated to dryness *in vacuo* at room temperature. The residue was extracted with ethanol and concentrated *in vacuo*. The same treatment was carried out as a control with the enzyme preparation without added riboflavin. Each of these concentrates was chromatographed on paper in three different solvent systems (5) and the chromatograms were developed for ortho- and organophosphate (6). The pattern of phosphate spots from both concentrates was almost identical in R_F values, spot areas, and color intensity. This fact, along with washing experiments described later, suggests the non-involvement of phosphate in the cleavage of riboflavin by the hydrolase.

Ribitol—The formation of ribitol as a product of the enzyme action was demonstrated by paper chromatographic analysis and by the isolation of ribitol as the dibenzylidene derivative.

Paper Chromatography—A portion of each of the ethanol extracts described above was applied as a spot to a strip of filter paper and chromatographed (ascending) successively in three solvent systems, *e.g.* 98 per cent pyridine, methanol, and 50 per cent methanol.

The first solvent separated ribitol from Tris buffer which is sensitive to some of the reagents used to detect ribitol, the second separated ribitol from riboflavin and lumichrome, and the third from a trace of an unidentified fluorescent substance. Table II shows the R_F values of the substances detected. In the enzyme control which had received no riboflavin, only the Tris buffer spot was found. The (presumed) ribitol spots from the reaction mixture displayed the same color upon development (7, 8) and possessed the same R_F values in the different solvents as authentic ribitol run concurrently on a control paper strip.

Isolation of Ribitol As Dibenzylidene Derivative—3 mg. of riboflavin were

[1] The substance emitting violet fluorescence was extracted from the paper and its absorption maximum was found to be 307 to 310 mμ in pyridine.

decomposed in a mixture consisting of 7 ml. of enzyme solution and 0.5 ml. of Tris buffer in a total volume of 37.5 ml. The enzyme reaction was stopped by adding 5 ml. of acetic acid. The clear supernatant fluid mixed with washings of the precipitate was extracted with ether overnight

TABLE II

Isolation of Ribitol by Successive Paper Chromatography

Enzyme preparation (13 ml.), 0.2 mM riboflavin (10 ml.), and 0.02 M Tris buffer were mixed and incubated for 4 hours. A control mixture containing no riboflavin was run simultaneously and analyzed in parallel as described below. After removal of material precipitated by acetic acid-alcohol, the supernatant fluid was concentrated *in vacuo*. The first paper strip chromatography was developed in 98 per cent pyridine; a guide strip spotted with a solution containing a mixture of ribitol, Tris buffer, lumichrome, and riboflavin was run concurrently. The portion of the chromatogram between R_F 0.8 and 1.0 was cut off, and the elutable material was concentrated and rechromatographed in methanol; a guide strip spotted with a solution containing a mixture of ribitol, lumichrome, and riboflavin was run concurrently. The portion of the second chromatogram between R_F 0.35 and 0.48 was cut off, and the elutable material was concentrated and chromatographed a third time in 50 per cent methanol; a guide strip spotted with ribitol was run concurrently. A portion of the strip between R_F 0.58 and 0.73 was cut off and the elutable material was concentrated and applied as a spot to a strip of filter paper. The strip was divided lengthwise in two parts. One part was sprayed with alkaline $KMnO_4$ solution (7) and the other with boric acid-bromocresol purple reagent (8). The presence of ribitol was detected in the chromatogram made from the riboflavin-enzyme mixture and in the guide strip containing authentic ribitol. No ribitol could be detected in the chromatograms made from the control enzyme mixture to which no riboflavin had been added.

Substance	R_F values in successive different solvents		
	98 per cent pyridine	Methanol	50 per cent methanol
Ribitol	0.86	0.43	0.66
Tris buffer	0.73		
Lumichrome	0.90	0.24	
Riboflavin		0.12	
Violet fluorescent substance	0.80–0.95	0.41	0.46
Green " "		0.19	

in a Kutscher-Steudel liquid-liquid extraction apparatus. Following the concentration of the aqueous residue *in vacuo*, the concentrate was mixed with 1.25 gm. of pulverized barium hydroxide and allowed to stand at 4° for 2 hours, followed by the addition of 45 ml. of 96 per cent ethanol. The mixture was kept overnight at 4°. In this procedure sugars and other impurities are eliminated (9). The precipitate thus formed was centrifuged

and the clear supernatant fluid was flushed with a stream of carbon dioxide to precipitate the barium in the system. After concentration of the supernatant liquid *in vacuo*, it was chromatographed as a band on paper with a guide strip of authentic ribitol. The solvent was methanol. The area of the paper corresponding to the authentic ribitol spot, as established by spraying the guide strip with alkaline $KMnO_4$ (7), was cut out and eluted repeatedly with ethanol. The combined eluates were evaporated to dryness *in vacuo*.

The amorphous white residue (which still contained small amounts of Tris buffer) was thoroughly dried in a vacuum desiccator and dissolved in 2 ml. of absolute methanol containing 0.01 ml. of freshly distilled benzaldehyde. Dry hydrogen chloride gas was slowly passed through the methanol solution for 15 minutes. The reaction was carried out in an ice bath to prevent any rise in temperature and the openings were blocked with tubes of dry $CaCl_2$ to keep the system moisture-free. The reaction mixture thus treated was evaporated *in vacuo* without heating and dried in a desiccator containing sodium hydroxide pellets until the mixture was free of hydrochloric acid. The use of 50 per cent H_2SO_4 in this synthesis, as reported by Fischer (10) and by Hackman and Trikojus (11), was unsatisfactory for minute amounts of ribitol.

White crystals predominated in the residue and they were washed with distilled water to remove the contaminating Tris buffer. After drying, the crystalline material sublimed slowly on a micro heating block at about 160°. The silk-like needles thus obtained in minute amount had the same shape and sublimation temperature (164–166°) as those of the authentic dibenzylidene derivative of ribitol prepared by the same procedure. No change in sublimation temperature was found with mixed crystals of the isolated and authentic material.

Characteristics of Riboflavin Hydrolase Action

Non-Involvement of Phosphate—An experiment in which paper chromatographic analysis for phosphate turnover in the reaction mixture revealed no apparent involvement of that group was previously described. Corroboration of this conclusion was obtained by use of the particulate enzyme-containing fraction which was washed repeatedly with cold Tris buffer (0.01 M) until no inorganic phosphate could be detected in the washings by means of the Fiske-Subbarow reagents (12). Inorganic phosphate and adenosine triphosphate were tested in separate aliquots of the enzyme preparation for their influence on the course of riboflavin hydrolysis. As shown in Table III, the enzyme was not significantly less active in the absence of added phosphate compounds than in their presence.

Oxygen Uptake—Oxygen uptake was measured in the conventional way in Warburg respirometers during the course of riboflavin hydrolysis. There was no difference in oxygen uptake with or without added riboflavin, indicating that the attack on riboflavin is non-oxidative and that the products of the primary hydrolysis are not further oxidized.

Inactivation by Anaerobiosis—Riboflavin hydrolase is greatly inhibited

TABLE III

Effect of Phosphates on Riboflavin Hydrolase Activity

Aliquots of the enzyme preparation (0.25 ml.) washed five times, 2.66×10^{-4} M riboflavin (0.25 ml.), and 0.025 M phosphate buffer or 2×10^{-3} M adenosine triphosphate (0.05 ml.) were mixed and shaken for 85 minutes in a water bath at 32°. Initial riboflavin concentration was 1.21×10^{-4} M.

Phosphate added	Final riboflavin concentration	Per cent decomposition
	$M \times 10^{-5}$	
None	3.59	70
Inorganic phosphate	1.57	87
Adenosine triphosphate	2.69	78

TABLE IV

Effect of Anaerobiosis on Riboflavin Hydrolase Activity

The mixture of enzyme preparation (0.25 ml.) and 2.66×10^{-4} M riboflavin (0.25 ml.) was incubated with shaking for 3.5 hours. The aerobic reaction was conducted in an ordinary test-tube and the anaerobic reaction was conducted in an evacuated Thunberg tube. Initial riboflavin concentration was 1.33×10^{-4} M.

Treatment	Final riboflavin concentration	Per cent decomposition
	M	
Aerobic	2.13×10^{-5}	84
Anaerobic	1.13×10^{-4}	15

in the absence of oxygen. One reaction was conducted in a Thunberg tube exhaustively evacuated while the control was run in an ordinary test-tube in an atmosphere of air. As presented in Table IV, a marked inhibition (82.1 per cent) was observed in the anaerobic reaction. This phenomenon will be discussed later.

Some Kinetics of Riboflavin Hydrolase

Effect of Substrate Concentration—Rates of enzyme activity in various concentrations of riboflavin were measured. As shown in Fig. 2, a striking

inhibition of the enzyme was observed at riboflavin concentrations greater than 5×10^{-5} mole per liter (18.8 mg. per liter). This concentration of riboflavin is considerably less than that required for induction of synthesis of riboflavin hydrolase by intact cells (30 mg. per liter). Very likely permeability properties of the cell wall contribute to the difference.

Effect of pH—Fig. 3 shows that the optimal pH for activity of the enzyme

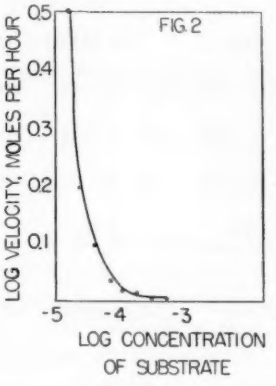


Fig. 2

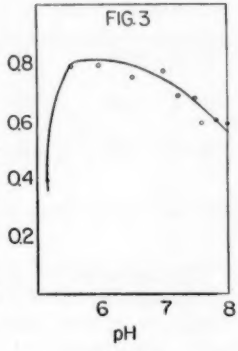


Fig. 3

Fig. 2. Effect of substrate concentration on the activity of riboflavin hydrolase. The systems consisted of 0.5 ml. of riboflavin solution, 0.5 ml. of Tris buffer (pH 7.0), and 0.2 ml. of enzyme preparation. The reaction was stopped in each system after 60, 120, 130, 140, and 210 minutes, respectively, for the concentrations of $10^{-4.78}$, $10^{-4.68}$, $10^{-4.38}$, $10^{-4.18}$, and more than $10^{-3.98}$ M riboflavin. The final concentration in each system was then measured.

Fig. 3. Effect of pH on the activity of riboflavin hydrolase. The systems consisted of 1 ml. of 0.067 M phosphate buffer, 0.1 ml. of 2.66×10^{-4} M riboflavin, and 0.1 ml. of enzyme preparation. The reaction was stopped after 40 minutes and the residual riboflavin was measured. The velocity of riboflavin hydrolysis (Figs. 2 and 3) was calculated on the basis that the reaction is monomolecular, since this was proved to be true at a fixed substrate concentration.

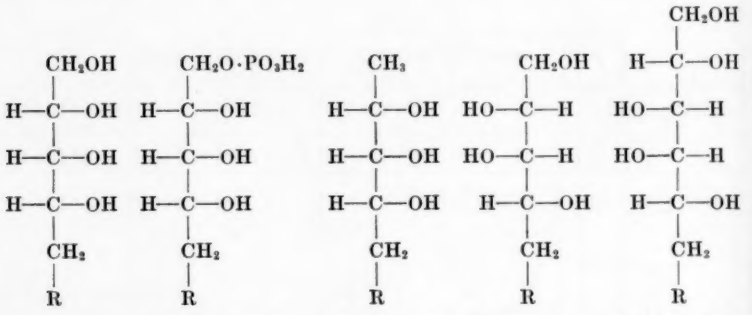
is fairly broad, *e.g.* pH 5.8 to 7.0. The optimum for riboflavin-decomposing activity of intact cells of *P. riboflavina* was reported to be pH 7.7 (1).

Specificity of Riboflavin Hydrolase

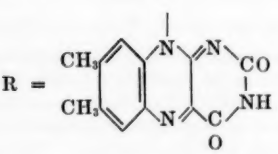
The specificity of the enzyme preparation was tested by using a limited number of compounds readily available. The compounds tested represent three classes: (1) different polyhydric alcohols substituted for ribitol in the riboflavin molecule, (2) different substitutions in the isoalloxazine ring,

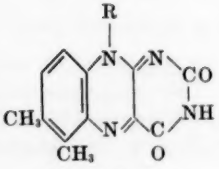
and (3) different *N*-ribityl-linked ring nuclei. Their structures are depicted in the accompanying forms.

Activity of the enzyme on all the compounds except the benzimidazole derivatives was tested fluorometrically, since the fluorescence of these compounds was considerably stronger than that of the corresponding products.

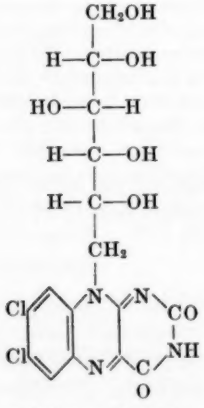


D-Riboflavin | D-Riboflavin-5′-phosphate | 5′-Deoxy-riboflavin | L-Lyxoflavin | D-Galactoflavin

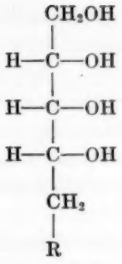




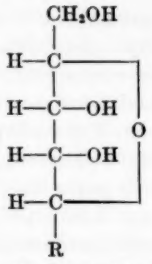
Isoriboflavin
R = ribityl



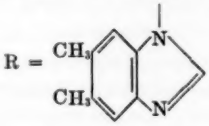
Dichlorosorboflavin



Ribityldimethylbenzimidazole



Ribosidodimethylbenzimidazole (α-ribazole)



Enzyme activity on the benzimidazole derivatives was tested by paper chromatography as described later.

Table V shows the relative activities of the enzyme on seven isoalloxazine compounds. It is noteworthy that riboflavin-5′-phosphate, the biologically active form of the vitamin, was hardly attacked by the enzyme, whereas it was rapidly oxidized by intact cells. In the latter, the possibility must be considered that phosphatase action liberated free riboflavin. Also, the possibility cannot be excluded that slight phosphatase activity existed in the cell-free enzyme preparation, or that a small amount of free riboflavin was present as an impurity in the riboflavin phosphate, thereby accounting for the slight apparent activity. Special experiments designed to see whether riboflavin phosphate inhibited riboflavin hydrolase competitively failed to reveal any inhibition at a concentration 10 times that of substrate riboflavin.

Blockage of the terminal alcohol group either by phosphorylation (riboflavin phosphate) or by reduction, *e.g.* the substitution of the terminal methylol by a methyl group (5′-deoxyriboflavin),[2] renders riboflavin as an unfavorable substrate for the enzyme. The configuration of polyhydric groups also seems to be one of the important factors for the hydrolyzing activity, since the 5-carbon lyxityl derivative, lyxoflavin,[3] was hydrolyzed at only 11 per cent the rate that riboflavin was hydrolyzed. Also, the 6-carbon polyhydric substituted compound, *e.g.* galactoflavin,[3] was not hydrolyzed detectably, but the 6-carbon sorbityl derivative, dichlorosorboflavin,[3] was hydrolyzed almost half as rapidly as riboflavin. Perhaps the

[2] We are indebted to Dr. D. Hendlin of Merck and Company, Inc., for supplying this compound.

[3] We wish to thank Dr. K. Folkers of Merck and Company, Inc., for supplying these compounds.

configuration around the 2′- and 3′-carbon atoms is vital for maximal enzymatic activity. Isoriboflavin,[4] a riboflavin antagonist in the rat (13), was hydrolyzed very slowly by the enzyme.[5]

The ribityl and the ribosido derivatives of benzimidazole, ribityldimethylbenzimidazole (1-(1′-D-ribityl)-5,6-dimethylbenzimidazole) and α-ribazole (1-(1′-D-ribosido)-5,6-dimethylbenzimidazole), were tested for hydrolysis by the enzyme by paper chromatographic detection of the expected product of hydrolysis, *e.g.* 5,6-dimethylbenzimidazole.

5 ml. of enzyme preparation, 0.5 ml. of 0.1 per cent ribityldimethylbenzimidazole[3, 4] or ribosidodimethylbenzimidazole[3] solution, and 0.5 ml. of

TABLE V

Specificity of Riboflavin Hydrolase

Experiments I to III were performed with different batches of enzyme preparations. In each experiment 0.2 ml. of enzyme preparation, 0.2 ml. of 10^{-3} M substrate, and 1.6 ml. of Tris buffer (pH 7.0) were mixed and shaken for 3 hours at 32°. Relative activity is expressed in terms of rate of decomposition of riboflavin which is assigned a value of 100.

Substrate	Experiment I		Experiment II		Experiment III	
	Per cent decomposition	Relative activity	Per cent decomposition	Relative activity	Per cent decomposition	Relative activity
D-Riboflavin	52	100	94	100	78	100
D-Riboflavin-5′-phosphate	3	6	13	14		
5′-Deoxyriboflavin	4	8				
L-Lyxoflavin			10	11		
D-Galactoflavin			0	0		
D-Dichlorosorboflavin					30	39
D-Isoriboflavin					6	8

0.067 M phosphate buffer (pH 7.0) were mixed and shaken at 32° for 5 hours. The reaction was stopped by the addition of 10 ml. of ethanol, and the precipitate formed was removed by centrifugation. The supernatant fluid was evaporated to dryness *in vacuo*, and the residue was extracted with 15 ml. of hot ethanol containing 0.1 ml. of ammonium hydroxide. The

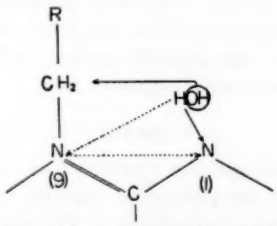
[4] We wish to thank Dr. J. A. Aeschlimann of Hoffmann-La Roche, Inc., for supplying these compounds.

[5] The end-product of enzyme hydrolysis of isoriboflavin, isolumichrome (5,6-dimethylalloxazine), was isolated by ether extraction of the reaction mixture. No evidence could be found in the literature that this particular alloxazine compound has been previously synthesized. It showed absorption maxima at 264 and 300 mμ, which are identical with those of isolumichrome synthesized chemically by an adaptation of the procedure described by Karrer *et al.* (4) for synthesis of substituted alloxazines.

extract thus obtained was concentrated and chromatographed (ascending) on a paper strip with 95 per cent isopropyl alcohol as solvent. A guide strip of authentic 5,6-dimethylbenzimidazole (Eastman) was also run in parallel. Several portions of the paper near the corresponding dimethylbenzimidazole spot on the guide strip (R_F 0.69) detected by ultraviolet illumination were cut out, eluted with ethanol, and adjusted to 0.01 N with HCl. The absorption spectrum of the eluates from each area did not show the typical absorption maxima of 284, 274, and 277 mμ of authentic 5,6-dimethylbenzimidazole (14), but showed the same absorption spectrum as that of the control experiment without added substrates. Thus, no dimethylbenzimidazole was present, indicating that the substrates were not hydrolyzed. It may be concluded that the *N*-ribityl group on the benzimidazole ring does not confer the specificity for riboflavin hydrolase that the *N*-ribityl group on the isoalloxazine ring does.

DISCUSSION

Demonstration of riboflavin hydrolase permits one to conclude that the first step in the attack of riboflavin by living cells of *P. riboflavina* (1, 2) is a hydrolysis followed by utilization of the ribitol, as contrasted to a piecemeal degradation of the ribityl chain still attached to the heterocyclic nucleus. It is possible that a similar hydrolytic reaction explains the production of lumichrome from riboflavin when a solution of the latter is exposed to daylight or sunlight. Karrer *et al.* (4) obtained a weak test for pentose in the irradiated mixture; the pentose may have been an irradiation product of initially formed ribitol.



The enzymatic hydrolytic cleavage of riboflavin conceivably could yield lumichrome (6,7-dimethylalloxazine) either directly or indirectly via its tautomer, 6,7-dimethylisoalloxazine (see the accompanying diagram).

In the former case the proton from water is directly combined with N atom 1 as shown by the solid arrow. In the latter case the proton would first react with N atom 9 to form the unstable isoalloxazine compound which then would undergo a tautomeric shift to lumichrome (broken arrows). These experiments shed no light on details of the reaction mechanism.

The inhibition of the enzyme by anaerobic conditions might be a reflection of the oxidation-reduction potential of the system. This effect has been observed with the hydrolytic enzymes (trypsin, chymotrypsin, β-amylase, urease) (15) and with oxalic acid decarboxylase (16).

The action of riboflavin hydrolase seems to be comparable to that of nucleosidase. Some of the nucleosidases are known to catalyze phosphorolytic reactions and some others catalyze hydrolytic reactions (17). Riboflavin hydrolase action is similar to the latter. Important to determine is whether the action of this enzyme is reversible. For example, the equilibrium in the phosphorolysis of inosine favors the synthesis of the nucleoside (18).

An alternative pathway of the biosynthesis of nucleotides has been postulated. Here the purine ring is completed after the introduction of the ribose phosphate moiety (19). In the biosynthesis of riboflavin, both pathways need also to be considered, *e.g.* the combination of a ribityl or a phosphorylated ribityl group with a completed alloxazine ring (lumichrome) and the introduction of a ribityl (or phosphorylated ribityl) group into an intermediary open chain compound prior to the formation of the heterocyclic compound. An attempt was made to demonstrate the reversibility of riboflavin hydrolase by using radioactive lumichrome and pure ribitol. This labeled compound was produced enzymatically from radioactive riboflavin extracted from a culture of the mold *Eremothecium ashbyii* (20), cultivated in a medium containing C^{14}-labeled acetate. The reversal experiment was unsuccessful because of the low specific activity of the labeled lumichrome preparation. However, it may be concluded that the action of this enzyme greatly favors the hydrolysis rather than the synthesis of riboflavin, since no riboflavin spot on the paper chromatogram of the reaction mixture could be detected after exposure of lumichrome and ribitol to the enzyme for a considerable period of time.

SUMMARY

An enzyme which catalyzes the hydrolysis of riboflavin to lumichrome and ribitol was found in the particulate fraction of ultrasonic extracts of the bacterium, *Pseudomonas riboflavina*. The enzyme was named riboflavin hydrolase. Lumichrome was characterized by its R_F values, its fluorescence, and its ultraviolet absorption spectrum. Ribitol was characterized by paper chromatography and also isolated as the dibenzylidene derivative. The enzyme was highly specific for riboflavin, attacking riboflavin-5′-phosphate very slowly. Most of the other isoalloxazine compounds tested were hydrolyzed only slightly. Indications are that a ribityl substitution in the N atom 9 of isoalloxazine is essential for riboflavin hydrolase activity. The riboside was not attacked nor was 1-D-ribityl-

5,6-dimethylbenzimidazole. Neither orthophosphate nor adenosine triphosphate was essential for the activity of the enzyme. Other characteristics of the enzyme are described.

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PENTACHLOROPHENOL AND MITOCHONDRIAL ADENOSINETRIPHOSPHATASE*

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The ability of dinitrophenol to uncouple oxidative phosphorylation and elicit ATPase[1] activity in freshly isolated, undamaged rat liver mitochondria is well established (1–3). In a previous report (4), evidence was presented which demonstrated similar properties for pentachlorophenol. Subsequent investigation has revealed that PCP, although evoking latent ATPase activity in undamaged mitochondria, in addition, and in striking contrast to DNP, inhibits the ATPase activity of damaged mitochondria.

In view of the importance of mitochondrial ATPase in its relationship to oxidative phosphorylation, it was of interest to study in more detail the effect of PCP upon mitochondrial enzymes which catalyze the hydrolysis of ATP. This report summarizes such studies and shows that PCP, unlike DNP, inhibits the ATPase activity in a variety of mitochondrial preparations, including "soluble" systems.

EXPERIMENTAL

Tissue Preparations—The mitochondria were isolated from rat liver by a slight modification (4) of the procedure of Schneider (5). Each ml. of the final mitochondrial suspension in 0.25 M sucrose represented 0.5 gm. of fresh liver. The suspension was used immediately in all experiments where undamaged mitochondria were desired.

Pre-aged mitochondria were prepared by incubation of the final mitochondrial suspension, diluted so that each ml. represented 0.33 gm. of fresh liver, at 37° for 20 minutes prior to its use in the reaction mixture.

Sonic disruption of the mitochondria was accomplished by exposing the final mitochondrial suspension, diluted so that each ml. represented 0.25 gm. of fresh liver, to the oscillations of a 9 kc. Raytheon oscillator for 10 min-

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† With the technical assistance of C. Elwood Claggett.

[1] The following abbreviations are used: ATPase, adenosinetriphosphatase; ATP, adenosine triphosphate; PCP, pentachlorophenol; DNP, 2,4-dinitrophenol. ATPase is used in this report to designate the enzyme or enzymes in the mitochondrial preparations which release inorganic phosphate from added ATP.

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utes at 2°. The suspension was then diluted further with an equal volume of 0.25 M sucrose. This preparation was used directly in the experiments of Table I. If "soluble" enzymes were desired, the suspension was centrifuged at 20,000 × *g* for 30 minutes at 0° and the opalescent, pale yellow supernatant fluid was used. This preparation was optically clear when examined under the phase contrast microscope. The pellet that remained after centrifugation also possessed ATPase activity, but was not used in the present study.

Benzene-treated mitochondrial preparations were obtained by adding sufficient benzene to the final mitochondrial suspension, diluted so that each ml. represented 0.125 gm. of fresh liver, to yield a concentration of 0.5 per cent by volume, after which the suspension was shaken manually. These preparations were used in the reaction mixtures 2 minutes after the addition of benzene.

The specific ATPase of liver mitochondria was prepared by the method of Kielley and Kielley (6). The sedimented red-brown gel was suspended in sufficient 0.25 M sucrose to yield an average N content of 0.2 mg. per ml. (micro-Kjeldahl).

A soluble ATPase preparation was obtained from acetone-desiccated mitochondria essentially according to the procedures (3, 7) described by Lardy and coworkers. A clear supernatant solution was obtained by centrifugation of the enzyme extract at 20,000 × *g* for 30 minutes at 0°.

Materials and Methods—ATP disodium salt (Pabst Laboratories) was used; PCP, DNP, and the other substituted phenols (Eastman) were recrystallized from aqueous alcohol. Solutions of all reagents were at pH 7.4 before addition to reaction mixtures. ATPase activity was determined in 13 × 100 mm. test-tubes as follows. The chilled tubes, which contained the reaction components, were immersed in a constant temperature bath, and the reaction was started by the addition of enzyme. Inorganic phosphate was determined (8) on aliquots of the reaction mixture after deproteinization with cold perchloric acid (final concentration, 2.5 per cent). The net release of phosphate was calculated from the differences between the inorganic phosphate content of the experimental reaction mixtures and the corresponding zero time values. Precautions were taken to assure proportionality to both enzyme concentration and time. All determinations were carried out in duplicate, and the results are average values of at least two separate experiments.

RESULTS AND DISCUSSION

Experiments with Intact Mitochondria—Freshly prepared 0.25 M sucrose suspensions of rat liver mitochondria are known to cause little, if any, phosphate release when incubated with ATP (3, 4, 9, 10). Actually, with most

of our preparations, a slight decrease of phosphate was observed when fresh mitochondrial suspensions were incubated with ATP. When PCP or DNP was added in the appropriate concentration, there was a marked phosphate release as shown in Fig. 1. At the lowest concentration of the substituted phenols (5×10^{-6} M), PCP was roughly twice as effective as DNP in evoking ATPase activity. However, as the concentrations of the substituted phenols were increased (5×10^{-5} M), the effectiveness of the compounds was essentially parallel, but at the higher concentrations (5×10^{-4} M and above) the ability of PCP to elicit the ATPase reaction fell off markedly, while that of DNP remained high. In view of this unexpected finding, it

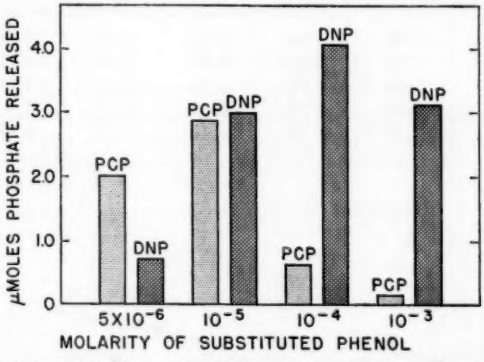

FIG. 1. Elicitation of ATPase activity from undamaged mitochondria. Each tube contained 0.04 M glycylglycine buffer, pH 7.4, 0.005 M $MgCl_2$, 0.006 M ATP, 0.2 ml. (0.40 mg. of N) of mitochondria plus substituted phenols as indicated, and sufficient 0.25 M sucrose to make a final volume of 1.0 ml. Incubation time, 10 minutes; temperature, 25°.

seemed of interest to test the effect of PCP upon the ATPase activity that could be released from mitochondria in other ways.

Experiments with Damaged Mitochondria—The three procedures used for releasing latent ATPase activity (Table I) were (1) pre-aging of mitochondria, (2) sonic disruption at 2°, and (3) treatment of the mitochondrial suspension with benzene. In all three cases of mitochondrial damage, PCP in concentrations of 5×10^{-4} M and higher strongly inhibited the phosphatase activity (Table I). DNP, on the other hand, was not inhibitory, even at the highest concentration employed (5×10^{-3} M), but stimulated rather slightly the hydrolysis of ATP.

Studies with "Soluble" Enzymes—These procedures are similar to those reported to increase the permeability of mitochondria (11). In order to evaluate a possible effect of permeability, "soluble" ATPase enzymes were

prepared from acetone-dried or sonically disrupted mitochondria. Furthermore, in view of the finding of Lardy and Wellman (3) that DNP

TABLE I

Effect of PCP and DNP on ATPase Activity of Damaged Mitochondria

Except for the enzyme source, the conditions of the experiment were those given in Fig. 1. Average N per tube, pre-aged mitochondria, 0.30 mg., sonically treated and benzene-treated, 0.11 mg. All the values are expressed as micromoles of phosphate released in 10 minutes at 25°.

Molarity	Pre-aged		Sonically treated		Benzene-treated	
	PCP	DNP	PCP	DNP	PCP	DNP
0	1.86	1.86	2.74	2.74	2.96	2.96
5×10^{-6}	1.89	2.00	3.08	2.85	2.90	3.30
5×10^{-5}	1.78	1.97	2.34	3.02	3.09	3.38
5×10^{-4}	0.38	2.11	0.62	3.25	1.13	3.62
5×10^{-3}	0.21	1.86	0.12	3.34	0.10	3.72

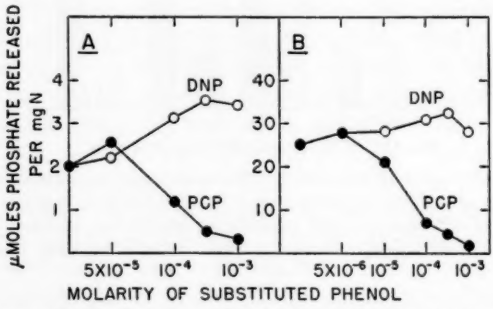

FIG. 2. Effect of PCP and DNP on "soluble" mitochondrial ATPase. *A*, acetone powder extract. Each tube contained 0.04 M glycylglycine buffer, pH 7.4, 0.001 M $MgCl_2$, 0.006 M ATP, and 0.3 ml. (0.74 mg. of N) of extract of acetone powder plus substituted phenols as indicated, and sufficient 0.15 M KCl to make a final volume of 1.0 ml. *B*, supernatant fluid from sonically disrupted mitochondria. Each tube contained the reaction mixture listed in Fig. 1, except that the enzyme source was 0.2 ml. (0.006 mg. of N) of supernatant fluid from sonically disrupted mitochondria. Substituted phenols as indicated were added, plus sufficient 0.25 M sucrose to make a final volume of 1.0 ml. Incubation time, 10 minutes; temperature, 25°.

enhanced the ATPase activity of soluble extracts of acetone-desiccated mitochondria, it was of interest to test PCP in such a system. The data summarized in Fig. 2, *A*, confirm that DNP increases the rate of ATP hydrolysis by the soluble extracts (3) and show, in addition, that PCP in low

concentrations (5×10^{-5} M) has a similar effect. However, PCP in concentrations of 5×10^{-4} M and higher inhibited, while DNP in corresponding concentrations enhanced, the dephosphorylation reaction.

Sonic treatment of 0.25 M sucrose suspensions of mitochondria released ATPase activity into the medium. The activity that remained in the supernatant fluid after centrifugation at 20,000 × *g* for 30 minutes at 0° was approximately 10 times higher, based on the total nitrogen content, than the activity of the acetone powder extracts (Fig. 2, *B*). It resembled the enzyme obtained from the extracts in that it could be sedimented from the supernatant fluid by centrifugation at 144,000 × *g* for 1 hour (*cf.* Lardy and Wellman (3)). Although the response to DNP was less than was obtained with acetone powder extracts, with PCP the ATPase activity again

TABLE II

Reversibility of PCP Inhibitory Action

The composition of the reaction mixture was the same as that given in Fig. 1. Pre-aged mitochondria served as the enzyme source. Incubated 10 minutes at 25° after washing. Other experimental details are given in the text.

Incubation time prior to washing	Per cent inhibition		
	Initial exposure to 5×10^{-4} M PCP prior to washing	After washing	Reexposure to 5×10^{-4} M PCP after washing
min.			
10	77.0	7.0	78.6
15	75.0	12.0	75.0

was markedly inhibited. The inhibition was even greater than that observed with the acetone preparation (Fig. 2, *B*).

Experiments with Reversibility—The reversibility of the PCP inactivation of mitochondrial ATPase was demonstrated with pre-aged mitochondria. If, after incubation with PCP, the mitochondria were removed by sedimentation, washed free of the drug,[2] and reincubated with fresh substrate, as shown in Table II, there was restoration of the ATPase activity. The mitochondrial enzymes were still capable of responding to the halophenol, for the addition of fresh PCP again caused marked inhibition of the ATPase reaction. As anticipated, with increasing time of exposure to PCP there was less reversal. With periods of exposure longer than 15 minutes or during which higher concentrations of PCP were used, there was significant loss of nitrogen into the medium.

Studies in Turbidity—The addition of high concentrations (5×10^{-3} M)

[2] Accomplished by suspending the mitochondria in fresh isotonic sucrose and recentrifuging at 8500 × *g* for 10 minutes at 0°. The washing was repeated once.

of PCP to 0.25 M sucrose suspensions of mitochondria caused a rapid and marked decrease in the turbidity of such suspensions (Fig. 3). At lower concentrations, the effect was slower and from a quantitative stand-point quite erratic, although decreases in turbidity were always observed. The decrease in turbidity was accompanied by a loss of nitrogen into the medium and release of acid-soluble substances, which absorb at 260 mμ (*cf.* Siekevitz and Potter (12)). This suggested swelling of the mitochondria and other alterations of mitochondrial structure. DNP, on the other hand, at concentrations as high as 5×10^{-3} M, had no effect on the measurements of

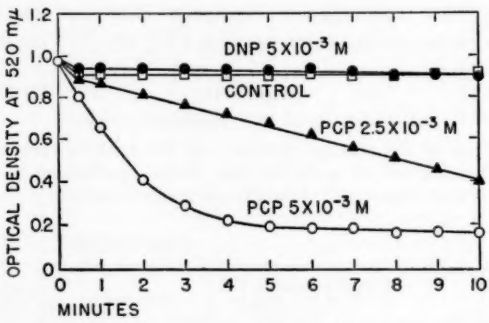

FIG. 3. Effect of PCP on the turbidity of mitochondrial suspensions. The cuvettes contained 0.04 M glycylglycine buffer, pH 7.4, 0.2 ml. (0.34 mg. of N) of mitochondria plus substituted phenols as indicated, and sufficient 0.25 M sucrose to make a total volume of 3.0 ml. At zero time either PCP, DNP, or an equal volume of H_2O (control) was added. Measurements were made in a Beckman model DU spectrophotometer at room temperature.

turbidity (Fig. 3). While this work was in progress, Hunter and Ford reported (13) on the ability of Versene[3] to protect mitochondrial suspensions from decreases in turbidity caused by surface-active agents such as saponin and deoxycholate. The presence of 0.01 M Versene in mitochondrial suspensions subsequently exposed to PCP not only failed to protect, but greatly accelerated the decrease in turbidity.

Other Halogenated Phenols—The inhibitory property of PCP for mitochondrial ATPase is shared by other halogenated phenols. As summarized in Table III, a reciprocal relation exists between the ability of a given halophenol to elicit ATPase and its ability to inhibit mitochondrial ATPase. Those phenols, which were most effective in releasing latent ATPase activity from intact mitochondria such as tribromo- and trichlorophenol, were the weakest inhibitors of ATPase of sonically treated mitochondria. The

[3] Sodium ethylenediaminetetraacetate.

2-chloro-4-nitrophenol resembles dinitrophenol in that it stimulated both types of ATPase activity. The dual effect of PCP was most evident at

TABLE III

Influence of Substituted Phenols on Two Types of ATPase Activity

Each tube contained the reaction mixture detailed in Fig. 1, plus other components as indicated. 0.2 ml. (0.31 mg. of N) of undamaged mitochondria served as enzyme source for the elicitation experiments and 0.2 ml. (0.056 mg. of N) of supernatant fluid from sonically disrupted mitochondria for the inhibition studies. Incubated for 10 minutes at 25°.

Phenol	Final concentration	ΔP, μM	
		Elicitation of ATPase (intact mitochondria)	Inhibition of ATPase (sonically disrupted mitochondria)
	M		
Control		0	1.72
Pentachloro-	5×10^{-4}	0.48	0.60
Pentabromo-	5×10^{-4}	0.30	0.34
2,4,6-Tribromo-	5×10^{-4}	1.83	1.52
2,4,6-Trichloro-	5×10^{-4}	2.37	1.46
2-Chloro-4-nitro-	5×10^{-4}	3.33	2.00
Pentachloro-	1×10^{-4}	2.07	0.96
Pentabromo-	1×10^{-4}	1.05	0.70
2,4,6-Triiodo-	1×10^{-4}	2.31	1.67

TABLE IV

Inhibitory Effect of PCP upon DNP Elicitation of ATPase

The conditions of the experiment were the same as those given in Fig. 1, except for the additions as noted. 0.2 ml. (0.34 mg. of N) of freshly prepared mitochondria served as enzyme source. Incubated for 10 minutes at 25°.

Incubation system	ΔP, μM
M	
Control	−0.15
DNP, 5×10^{-5}	+2.56
PCP, 5×10^{-4}	+0.61
DNP, 5×10^{-5} + PCP, 5×10^{-4}	+0.54

the lower concentration range. At 1×10^{-4} M, PCP caused a clear release of ATPase from intact mitochondria and still exerted a pronounced inhibition of the dephosphorylating activity of the supernatant fluid from sonically disrupted mitochondria (Table III).

PCP strongly inhibited the ATPase activity evoked by DNP. As shown

by the experiments summarized in Table IV, the addition of 5×10^{-5} M DNP to fresh mitochondria produced the usual marked ATPase activity, while 5×10^{-4} M PCP caused only a slight ATPase effect (*cf.* Fig. 1). However, when both compounds were added to the same reaction vessel, there was no summation of the two separate activities, but on the contrary a marked depression of the phosphate release from ATP occurred. It is probable that high concentrations of PCP inhibit the same ATPase enzyme or enzymes that low concentrations evoke. This is seen indirectly by the data of Table IV.

In order to examine the inhibitory effect of PCP upon a more specific mitochondrial ATPase, the enzyme described by Kielley and Kielley (6)

TABLE V

PCP Inhibition of Specific ATPase from Disintegrated Mitochondria

Each tube contained 0.04 M glycylglycine buffer, pH 7.4, 0.005 M $MgCl_2$, 0.004 M ATP, 0.2 ml. (0.04 mg. of N) of disintegrated mitochondria (6) plus substituted phenols as indicated, and sufficient 0.25 M sucrose to make a total volume of 1.0 ml. Incubation period, 15 minutes; temperature, 25°.

Concentration of substituted phenol	ΔP, μM	
	PCP	DNP
M		
0	3.50	3.50
5×10^{-5}	3.30	3.66
5×10^{-4}	2.08	3.80
5×10^{-3}	0.17	3.50

was prepared from disintegrated mitochondria. PCP in the higher concentrations exerted a strong inhibitory effect upon this enzyme also (Table V), while DNP had no significant effect. In view of the Mg^{++} activation of this enzyme and the possibility that PCP might inhibit by binding this metal, the effect of Mg^{++} on the PCP inhibition was studied. With a Mg^{++} concentration of 1×10^{-4} M, PCP at 5×10^{-4} M caused a 73 per cent inhibition. Upon increase of the Mg concentration to 5×10^{-3} M, the inhibition was 77 per cent. This indicates that removal of Mg^{++} by PCP seems unlikely.

Also, the inhibition by PCP appears to be independent of the substrate concentration. Experiments with acetone powder extracts revealed that the degree of inhibition caused by 5×10^{-4} M PCP was not changed when the ATP concentration was increased from 0.0015 M to 0.012 M.

To evaluate the rather remote possibility that pentachlorophenyl phosphate might be an intermediate in the ATPase reaction, this compound was

synthesized[4] and found to be inert in either intact or damaged mitochondrial systems.[5]

Effect on Other Phosphatases—A few experiments performed with phosphatases from other tissues indicated that PCP is not a general phosphatase inhibitor. Thus, in concentrations up to 5×10^{-3} M, PCP had no appreciable effect on human or rabbit alkaline serum phosphatases when determined by either the modified Bodansky method (15) with glycerophosphate as substrate or a procedure (16) in which *p*-nitrophenyl phosphate was employed as substrate. Furthermore, PCP at 5×10^{-4} M did not suppress the acid phosphatase of rat liver homogenate as estimated by the glycerophosphate method (15). Similarly, 5×10^{-4} M PCP did not alter the rate of hydrolysis of ATP or *p*-nitrophenyl phosphate in the presence of potato phosphatase[6] at pH 5.0.

While the concentrations of PCP which are most effective in uncoupling oxidative phosphorylation are less than those required for maximal inhibition of ATPase, there is some overlapping. Thus, PCP at 1×10^{-4} M almost completely suppressed oxidative phosphorylation (4), while in the same concentration the compound effected a 44 per cent inhibition of ATPase from damaged mitochondria (Table III). At this same concentration, ATPase was released from intact mitochondria, which illustrates the dual effect of PCP upon mitochondrial enzymes. In view of this dual effect, it seems unlikely that the uncoupling action of PCP is to be attributed solely to enhancement of ATP breakdown. Other mechanisms which involve mitochondrial structural changes (Fig. 3), as well as a direct effect on phosphate-transferring enzymes (possibly reflected here as inhibition of ATPase), seem more probable.

SUMMARY

Pentachlorophenol, in contrast to dinitrophenol, exerted a dual effect upon mitochondrial ATPase. In low concentrations (5×10^{-5} M), PCP elicited ATPase activity from freshly prepared, undamaged rat liver mitochondria and enhanced the hydrolysis of ATP by soluble preparations. In higher concentrations (5×10^{-4} M), PCP inhibited the ATPase activity of damaged mitochondria as well as the activity of the soluble preparations.

The mechanism of the inhibition is not attributable to chelation of Mg^{++};

[4] By the phosphorylation of PCP with $POCl_3$ essentially according to the procedure used by Bessey and Love (14) for the synthesis of *p*-nitrophenyl phosphate. Calculated for $C_6Cl_5O_4PNa_2 \cdot 2H_2O$, C 16.90, H 0.94, Na 10.79; found, C 16.89, H 1.00, Na 10.82. The theoretical amount of phosphate was liberated after refluxing with 1.0 N HCl for 1.5 hours.

[5] Compare the statement of Lardy and Wellman (3) concerning the stability of dinitrophenyl phosphate.

[6] We are indebted to Dr. G. Ashwell for a gift of this enzyme.

nor did it appear to be related to a non-specific inactivation of enzyme protein, since the reversibility of the ATPase inhibition could be demonstrated. Furthermore, other phosphatases are not inhibited by PCP.

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HEPATIC INTERVENTION IN THE BINDING OF ESTROGEN TO RAT SERUM ALBUMIN IN VITRO*

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(Received for publication, November 21, 1955)

Previous studies from these laboratories have demonstrated the presence of estrogenic lipoprotein complexes in the circulation of several species (*cf.* Szego and Roberts (1)). An enzymatic mechanism capable of promoting the binding of C^{14}-labeled estrone (2) or estradiol,[1] or their metabolites, to rat serum proteins *in vitro* was demonstrated in surviving rat liver tissue. Estrogen-serum protein binding capacity was correlated with the functional state of the liver (2, 3) and was absent in all the other tissues studied (2).

In the present investigations, paper electrophoresis and cold ethanol fractionation have been employed in an attempt to characterize the serum proteins which participate in the binding process *in vitro*. Under these circumstances, estrogen-serum protein binding in the presence of surviving rat liver tissue occurred selectively with albumin (4, 5). In addition, this association was inhibited or abolished by increasing concentrations of added cortisol (6).

Methods

The incubation techniques were similar to those described earlier (2). Each incubation vessel contained 2 ml. of freshly obtained rat serum in which were present 17.5 γ of estrone-16-C^{14}, equivalent to 36,300 c.p.m. determined on an automatic gas flow planchet sample changer.[2] To some of the vessels were added 250 mg. of rat liver or splenic tissue prepared by free-hand mincing. The vessels were incubated at 37.5° in a Dubnoff metabolic incubator for 2 hours, except when otherwise noted, under a constant stream of 95 per cent O_2-5 per cent CO_2. Where indicated, unlabeled cortisol[3] as the free alcohol was added in a total volume of 0.05 ml. of

* Aided by grants from the National Cancer Institute of the National Institutes of Health, United States Public Health Service (No. C-1488 and No. C-1408), and by Cancer Research Funds of the University of California.

[1] Szego, C. M., unpublished experiments.

[2] The isotopic estrogens were obtained from Charles E. Frosst and Company, Montreal, Canada.

[3] The generous contribution of cortisol by Dr. Richard H. Barnes of Sharp and Dohme, Division of Merck and Company, Inc., and Dr. Elmer Alpert of Merck and Company, Inc., is gratefully acknowledged.

propylene glycol. Controls for the cortisol experiments contained the equivalent volume of propylene glycol.

After incubation, the vessel contents were rapidly centrifuged in the cold, and 20 μl. samples were subjected to paper electrophoresis by either the horizontal open strip method[4] or by the hanging strip procedure of Durrum (7). Specific electrophoretic conditions for individual experiments are given in the legends of Figs. 1 to 6. The dried strips were analyzed for radioactivity at 0.5 cm. increments in an automatic gas flow strip counter. Reference standards of pure estrone-16-C^{14} evaporated from an alcoholic solution on paper strips, or added to serum in the usual fashion and placed on the paper, gave values for radioactivity in the strip counter approximating 50 per cent of that observed when aliquots of the same solutions were placed on planchets and counted in the automatic planchet changer. Absorption of radioactivity by the paper and a lower sensitivity of the strip counter were found to be responsible for this decrease. The values obtained on the strip counter and corrected for background were therefore multiplied by 2 so that the radioactivity plotted on the figures could be compared directly with the original counts present in the 20 μl. sample of incubation medium placed on the experimental strips. It may be calculated that the latter value was 363 c.p.m. Inspection of Figs. 1 to 3, in which all of the counts present on the complete strip are plotted, reveals that the total radioactivity recovered averaged about 200 c.p.m. when the values at 0.5 cm. increments were added. The major portion of the missing radioactivity was due to absorption by protein (about 15 per cent of total radioactivity added) and loss to the buffer wetting the paper during electrophoresis (about 15 per cent), as judged by control experiments with known amounts of isotopic steroid. Correction for these losses was not made during calculation of the data on radioactivity, since there was no way of determining the exact adjustment to be applied in each instance.

After counting, the strips were stained with bromophenol blue (7) and mounted beneath the curves representing distribution of radioactivity.

RESULTS AND DISCUSSION

The results obtained are depicted in Figs. 1 to 6, in which the curves represent radioactivity in counts per minute present in successive 0.5 cm. portions of the paper strips. The electrophoretic origin is indicated by zero on the abscissa.

[4] The authors are indebted to Dr. Harold A. Levey, Department of Physiological Chemistry, University of California, Los Angeles, for permission to cite his unpublished modifications of the horizontal open strip technique of paper electrophoresis utilized in some of the present studies.

Fig. 1 demonstrates the findings in the incubated serum control containing no tissue, subjected to the horizontal open strip method of electrophoresis. It will be observed that a single peak of radioactivity occurred on the strip, probably representing unchanged steroid. Its migration, just behind the slowest proteins, appeared to be due to the phenomenon of hydrodynamic displacement to which this method of electrophoresis is subject (*cf.* Durrum (7)), rather than to inherent mobility under these conditions. This suggestion was supported by dextran control experiments, as

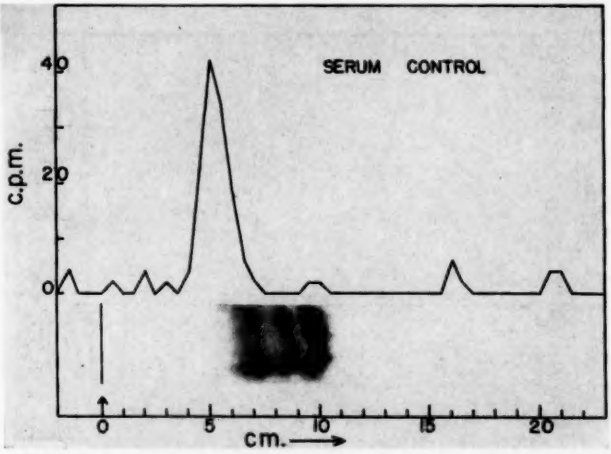

FIG. 1. Absence of protein-bound radioactivity after incubation of estrone-16-C^{14} in the presence of serum alone. See the text for the conditions of incubation and isotopic analysis. The horizontal open strip method of paper electrophoresis was used with Whatman No. 1 paper strips, 1½ inches wide. Electrophoresis was carried out in Veronal buffer, pH 8.6, $\mu = 0.1$, at a constant current of 1 ma. per cm. for 19 hours.

well as by failure of the peak of radioactivity to migrate beyond the electrophoretic origin in the Durrum hanging strip method applied to similar serum control samples (see below).

Fig. 2 reveals that a significant proportion of the total radioactivity migrated in the albumin area of the serum proteins when incubation was carried out in the presence of surviving rat liver tissue. It may be noted further that the location of *residual* (unbound) radioactivity coincided with that observed in the serum control (Fig. 1); *i.e.*, behind the γ-globulin.

Fig. 3 represents the results of a parallel experiment carried out with spleen, which, in common with kidney and uterus, had proved inactive in promoting the binding to the serum proteins of radioactivity derived from estrone-16-C^{14} in earlier studies (2). It will be observed that the results

are indistinguishable from those obtained with the serum control, and that no isotope was associated with any of the protein areas. Thus, these data fully confirm the specificity of a liver system capable of effecting estrogen-protein association *in vitro*.

Fig. 4 demonstrates the influence of incubation time on steroid-protein association in the presence of surviving liver tissue. In this and in Figs. 5 and 6, the electrophoretic patterns were obtained by the Durrum hanging strip procedure (7). It will be observed (Fig. 4, left side) that the peak of radioactivity in the protein areas coincided with albumin, as in

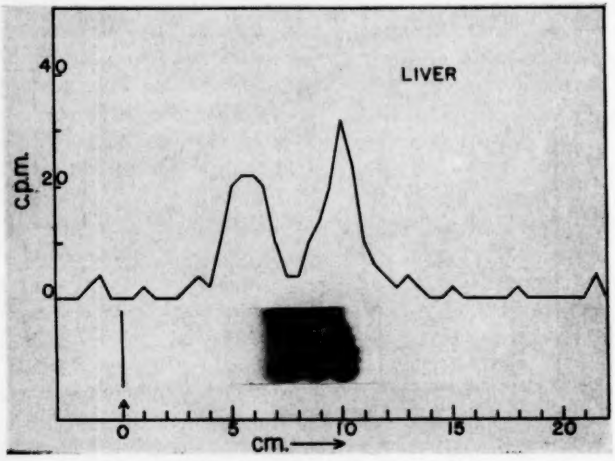

FIG. 2. The occurrence of albumin-bound radioactivity after incubation of estrone-16-C^{14} in the presence of surviving rat liver in a homologous serum medium. Experimental conditions as in the text and in Fig. 1.

the horizontal open strip method (Fig. 2). However, in the Durrum procedure, a large proportion of the residual (unbound) radioactivity remained at or near the electrophoretic origin. As the time of incubation was prolonged, the association of radioactivity with the albumin area increased and was virtually complete in 6 hours, with practically no activity retained at or near the origin. These observations were highly reproducible, occurred also when estradiol-16-C^{14} was substituted for isotopic estrone, and were confirmatory of the results obtained with the acetone precipitation techniques described earlier (2).

The following experiment was performed to determine whether the radioactive material associated with the albumin area on the paper strips retained estrogenicity. Estradiol-16-C^{14} was incubated for 2 or 6 hours in

rat serum in the presence of surviving rat liver tissue. Thereafter, five 20 μl. aliquots of each of the resultant serum media were subjected to the horizontal strip method of electrophoresis under conditions similar to those noted in Figs. 1 to 3. To achieve a resolution of albumin and α_1-globulin better than that obtained in the latter experiments, the buffer employed was similar to that described in the legend to Fig. 4, but was of $\mu = 0.08$ rather than 0.10. Immediately after electrophoresis, a single strip from each of the two time series was rapidly dried and stained for localization of protein distribution. During the time required for this procedure, the

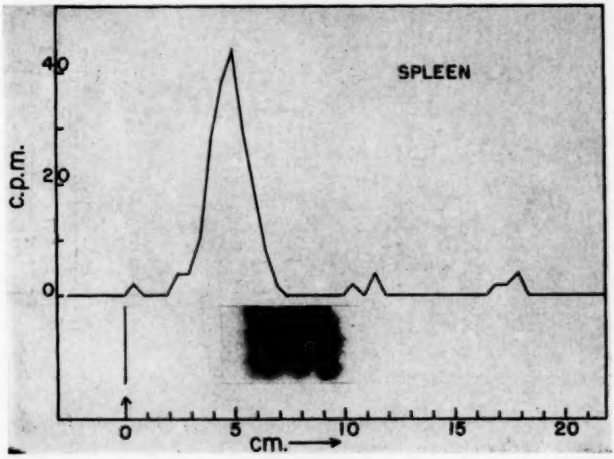

FIG. 3. Absence of protein-bound radioactivity after incubation of estrone-16-C^{14} in the presence of surviving rat spleen in a homologous serum medium. Experimental conditions as in the text and in Fig. 1.

remaining four strips in each group were rapidly frozen between sheets of aluminum foil on a slab of dry ice to minimize alteration of the protein patterns. The albumin areas of these strips were located with dividers after the migration distance from the origin and the width of the band had been determined from the reference strip. The albumin regions and certain other control areas similarly located were then cut from the frozen strips, further subdivided into small squares, and eluted by overnight shaking in the cold in the presence of distilled H_2O adjusted to pH 9.5. The location of the protein bands was subsequently confirmed by staining those portions of the electrophoretic strips not subjected to the elution process. After adjustment of pH to 4.6 with acetate buffer, the eluates were hydrolyzed (*cf.* Szego and Wolcott (8)) by incubation for 24 hours with beef

liver β-glucuronidase (Warner-Chilcott), neutralized, and assayed in immature rats by a modified Astwood procedure (*cf.* Szego and Roberts (1)). The semiquantitative results obtained revealed that approximately 40 per cent of the estrogenic activity originally placed on each 2 hour strip (0.18 γ) could be eluted from the areas analyzed. This was equally divided between the albumin area and the region corresponding to unbound radioactivity. Negligible estrogenic activity occurred in the α-globulin region.

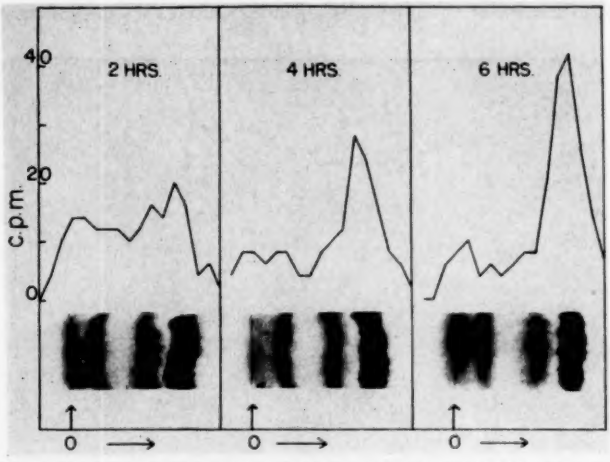

FIG. 4. The influence of time of incubation on liver-catalyzed binding to albumin of radioactivity derived from estrone-16-C^{14}. See the text for the conditions of incubation and isotopic analysis. The Durrum hanging strip technique of paper electrophoresis was used with Whatman No. 1 paper strips, 1½ inches wide. Electrophoresis was carried out in barbiturate buffer (Veronal, 0.05 M; sodium acetate, 0.0375 M; sodium chloride, 0.0125 M; $\mu = 0.1$; pH adjusted to 8.6) at a constant current of 0.5 ma. per cm. for 15 hours.

On each 6 hour strip approximately 25 per cent of the estrogenic activity originally added could be eluted from the albumin area. These data indicate that a significant portion of the steroid bound to serum albumin in the presence of rat liver tissue *in vitro* retained estrogenic activity. In contrast, metabolites of estrogen bound to liver proteins *in vitro* appear to be devoid of biological activity (*cf.* Szego and Roberts (3, 5) and Riegel and Mueller (9)).

The specific localization of isotopic estrogen or its metabolites in serum albumin under the present experimental conditions has been substantiated by an independent method. The serum medium was pooled from five vessels in which estrone-16-C^{14} had been incubated for 6 hours with surviving

liver tissue. This medium was subjected to cold ethanol fractionation by a modification[5] of the method of Ulrich, Li, and Tarver (10). The fractions obtained were lyophilized without prior dialysis to avoid loss of the steroid which is dialyzable under these conditions (11). 6 per cent solutions of the dried protein fractions were then made up in distilled water, and 20 μl. samples were subjected to paper electrophoresis by the Durrum technique. The results are shown in Fig. 5. It will be noted that radioactivity persisted in the albumin throughout the fractionation procedures. In-

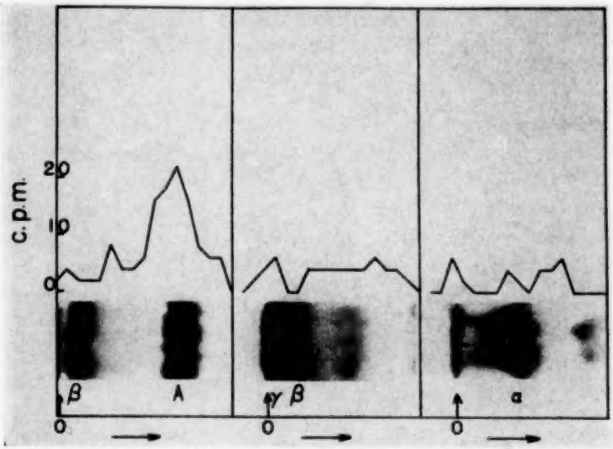

FIG. 5. The persistence of albumin-bound radioactivity through cold ethanol fractionation. See the text for details. The left-hand section of the chart represents Fraction V (albumin contaminated with about 10 per cent β-globulin); the middle section, Fraction II (principally β- and γ-globulins); the right-hand section, Fraction IV (α_1-globulin with a trace of denatured material at electrophoretic origin). Electrophoretic conditions as in Fig. 4.

significant amounts of radioactivity were associated with other principal or contaminating components of the several fractions. Whether or not endogenous estrogen is present in rat serum bound to albumin is unknown. It will be recalled that endogenous circulating estrogen in pooled human plasma occurred almost exclusively in the crude β_1-lipoprotein fraction as originally prepared by Cohn *et al.* (*cf.* Roberts and Szego (12)). It should be noted, however, that in animals other than man albumin may assume a lipide-transporting rôle in the absence of those β-lipoproteins peculiar to human plasma (*cf.* (13), and Morris and Courtice (14)).

The methods utilized in the present study have also proved applicable

[5] Roberts, S., and Kelley, M. B., unpublished observations.

to the examination of steroid interactions in the binding phenomenon. Earlier investigations from these laboratories (*cf.* Szego and Roberts (1)) had revealed profound estrogen-antagonizing effects of the 11-oxy, 17-hydroxyadrenocortical steroids, as evidenced by inhibition by the latter of gross and metabolic signs of estrogen-induced uterine growth in adult castrated rats. The suggestion was advanced that this phenomenon might be due to competition by the two classes of steroid for binding to proteins which function in steroid transport or in mediation of hormonal effects.

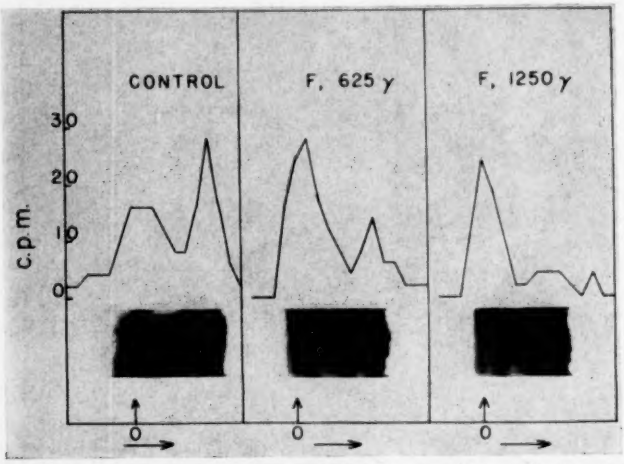

FIG. 6. The suppressing influence of cortisol (Compound F) on the liver-catalyzed binding to albumin of radioactivity derived from estrone-16-C^{14}. See the text for experimental conditions. Electrophoresis was carried out by the Durrum hanging strip technique on Whatman No. 3 MM paper strips, 3 cm. wide, at a constant current of 1 ma. per cm. for 5½ hours. Buffer as in Fig. 4.

This suggestion appears to gain support from the data in Fig. 6, which depicts the influence of increasing levels of unlabeled cortisol (Compound F) upon the liver-catalyzed association of isotopic estrone with rat serum albumin. It will be observed that 625 γ of cortisol significantly inhibited the degree of estroprotein formation seen in the control and that doubling the cortisol level virtually abolished the process. Similar data have been obtained with combinations of estradiol and 11-deoxycorticosterone. The large amount of cortisol required to prevent estrogen-protein binding *in vitro* (625 γ *versus* 17.5 γ) is in line with the *in vivo* observations (1) and suggests that the relative affinities of the two classes of compounds for serum protein are vastly in favor of the estrogens. The latter appear by

all criteria to be the most firmly bound of all the steroids thus far examined (*cf.* Roberts and Szego (5)).

Shortly after a brief description of the present studies appeared in abstract form (4), Westphal and coworkers reported physicochemical observations (15) on the binding of progesterone-4-C^{14} in the absence of tissue to rat and human serum albumin *in vitro*, as judged by essentially similar paper electrophoretic and radioisotopic analyses of human and rat sera and of 4 per cent human serum albumin solutions containing the isotopic steroid. Cortisol-4-C^{14} did not appear to be bound when judged by these criteria (15), but subsequent ultracentrifugal and free electrophoresis studies by Westphal (16) indicated that the major portion of this steroid associated rather weakly with human serum albumin *in vitro*. The specificity of the association, which occurred without hepatic intervention, was not established by extension of the study to other proteins.

The physiological significance of the binding *in vitro* of steroid hormones to serum albumin is unknown. It has been postulated, however, that the transport of these hormones in the body is dependent upon their association with serum protein (1). In the case of estrogen, at least, the liver appears to be essential for the binding process *in vitro* and for biological activity *in vivo* (17).

Miss Dorothy C. Wolcott, Mrs. M. B. Kelley, and Mr. Edwin M. Weller contributed valuable technical assistance.

SUMMARY

By paper electrophoresis and cold ethanol fractionation techniques, it has been demonstrated that surviving rat liver tissue catalyzes the binding of estrone-16-C^{14} and estradiol-16-C^{14} specifically to the albumin of a homologous serum medium. The bound steroid retained estrogenic activity. When serum containing estrone-16-C^{14} was incubated alone or in the presence of splenic tissue, binding did not occur. The enzymatically catalyzed association of estrone or its metabolites with albumin was inhibited or abolished by increasing concentrations of added cortisol. These findings substantiate earlier data from these laboratories on the specificity of the liver in effecting estrogen-serum protein association *in vitro* and offer an explanation for the competitive phenomena exhibited by the estrogens and certain corticosteroids *in vivo*.

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STRUCTURAL STUDIES ON THE NUCLEIC ACIDS OF SOME STRAINS OF TOBACCO MOSAIC VIRUS

I. THE RESIDUAL MATERIAL AFTER RIBONUCLEASE DIGESTION*

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(Received for publication, November 28, 1955)

It seems probable that the different disease symptoms caused by strains of tobacco mosaic virus (TMV) are reflected in chemical differences in the viral strains themselves. The search for such differences has led to the analysis of the nucleic acids and protein components of many strains. In some cases, but not in all, differences in protein composition were demonstrable (1). In no instances have unequivocal differences in composition of the nucleic acids been shown (2, 3), although there was orig nally some suggestion of this (4). From a consideration of all the analytical work, it has been concluded that the really critical differences among strains may be in structural features yet to be determined (1, 5).

Since ribonuclease appears to be a highly specific phosphodiesterase (6–8) which will hydrolyze only secondary phosphate esters of pyrimidine ribonucleoside 3′-phosphates, it seemed possible that this enzyme might be useful in probing the structural differences in viral nucleic acids. As a beginning, the composition was determined of the residual (trichloroacetic acid-precipitable) material after ribonuclease digestion of the nucleic acids of each of five strains of TMV. The ribonuclease-resistant residues were found to be virtually identical in composition.

Materials and Methods

Nucleic acids were isolated from the purified preparations of each virus according to the method of Cohen and Stanley (9) as modified by Knight (2).

The ribonuclease used in these studies was a crystalline preparation obtained from the Worthington Biochemical Corporation, Freehold, New Jersey.

Rate of Degradation of TMV Nucleic Acid with Ribonuclease—In order to determine suitable conditions for treating the nucleic acids of different

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strains with ribonuclease, a preliminary experiment was made with TMV nucleic acid.

100 mg. of nucleic acid were dissolved in 2 ml. of 0.1 M borate solution at pH 7.6. There was a drop in the pH when all the nucleic acid went into solution. The pH was readjusted to 7.6 with careful addition of ice-cold N NaOH, and the final volume was made up to 5 ml. with pH 7.6 borate. To this were added with mixing 150 γ of ribonuclease dissolved in 0.1 M borate at pH 7.6, and the mixture was held at room temperature (23°). At various intervals of time (Table I), 1 ml. aliquots were pipetted into 0.14 ml. of 50 per cent trichloroacetic acid (TCA), mixed well, held at 0° for 1 hour, and centrifuged in the cold. The residue was washed twice with 6 per cent TCA, once with 95 per cent alcohol, once with absolute alcohol, and finally dried *in vacuo* over P_2O_5 at 56.5°. The phosphorus content of each residue was determined according to the method of King (10), and from this the weight of residue by use of a factor based on the phosphorus content of the original nucleic acid. The results are shown in Table I, together with the molar proportions of the purine and pyrimidine bases which were determined with Wyatt's procedure (11).

TABLE I

Precipitability of TMV Nucleic Acid by 6 Per Cent TCA at Various Times during Treatment with Ribonuclease at pH 7.6 and 23° and Molar Proportions of Bases in TCA-Precipitable Material*

Time	Nucleic acid precipitated by TCA	Guanine	Adenine	Cytosine	Uracil	$\frac{\text{Purines}}{\text{Pyrimidines}}$
hrs.	*per cent*					
0	100	1.00	1.03	0.66	1.04	1.19
6	22	1.00	1.17	0.24	0.33	3.80
12	21	1.00	1.21	0.22	0.30	4.25
24	17	1.00	1.19	0.20	0.27	4.60
33	14	1.00	1.21	0.17	0.25	5.26

* Guanine is taken arbitrarily as 1.00.

From this preliminary experiment a digestion time of 36 hours was chosen for the reaction of the nucleic acids of different strains with ribonuclease.

Degradation of Nucleic Acids of Different Strains with Ribonuclease—30 mg. of the nucleic acids of five strains of TMV (2, 12, 13) were dissolved in borate at pH 7.6, as described above. The final pH and the volume of the solutions were 7.6 and 1.5 ml., respectively. To each were added with mixing 45 γ of ribonuclease dissolved in 0.1 ml. of 0.1 M borate at pH 7.6, and the mixtures were incubated at room temperature (23°). At the end of 36 hours, 1 ml. was pipetted into 0.14 ml. of 50 per cent TCA. The TCA-precipitable residues were washed and dried, and the purine and py-

rimidine compositions of the dry residues were determined as described above. The results are presented in Table II.

Sedimentation Behavior of Ribonuclease-Resistant Residue of TMV Nucleic Acid—One preparation of ribonuclease-resistant residue was prepared by treating TMV nucleic acid with ribonuclease for 36 hours as previously described. The residue, in the form of the TCA precipitate, was washed several times with alcohol and then with ether and finally dried *in vacuo* at room temperature. This material was dissolved in 0.18 M sodium chloride at a concentration of about 46 γ per ml., and its sedimentation behavior was kindly examined by Dr. H. K. Schachman, who employed a synthetic boundary cell and ultraviolet optics. The sedimentation constant of the material was about 0.9 S.

TABLE II

Molar Proportions of Bases in Material Precipitable from Nucleic Acids by 6 Per Cent TCA after Ribonuclease Digestion for 36 Hours at 23° and at pH 7.6*

Strain	Guanine	Adenine	Cytosine	Uracil	$\frac{\text{Purines}}{\text{Pyrimidines}}$
TMV	1.00	1.20	0.17	0.28	4.89
YA	1.00	1.16	0.17	0.28	4.81
HR	1.00	1.14	0.17	0.27	4.86
M	1.00	1.16	0.17	0.27	4.91
J14D1	1.00	1.20	0.17	0.28	4.89

* Guanine is taken arbitrarily as 1.00.

RESULTS AND DISCUSSION

Ribonuclease caused a considerable reduction in the TCA-precipitable portion of TMV nucleic acid within 6 hours, after which the reaction leveled off (Table I). After 33 hours of reaction, the amount of residue precipitable by 6 per cent TCA was 14.0 per cent of the original nucleic acid.[1] The residue was rich in purines and poor in pyrimidines. The content of adenine was higher than that of any of the other bases (Table I). This is in contrast to the ribonuclease-resistant residues of ribonucleic acids of pig liver and yeast which were found to be rich in guanine (15). However, as might be expected, the compositions of the residues are in all cases consistent with the compositions of the intact nucleic acids, for the yeast and pig liver nucleic acids contain more guanine than adenine, whereas this is not true for TMV.

The compositions of ribonuclease-resistant residues obtained after hy-

[1] This is less residue or "core" than reported by Holden and Pirie (14) for their TMV nucleic acid, but our reaction conditions were somewhat different from theirs and this probably accounts for the difference in results.

drolysis of the nucleic acids of five strains of TMV with ribonuclease for 36 hours were essentially the same (Table II). These results indicate some structural similarity in the nucleic acids of different strains, for it is difficult to explain the present results unless one assumes that the nucleic acids of different strains possess certain oligonucleotide segments of identical composition, and that the pyrimidine units are uniformly spaced therein. Within these oligonucleotide segments, the purines could, of course, be arranged in a variety of different sequences. The evaluation of this possibility awaits the application of suitable stepwise degradation methods.

It would be interesting to know more about the size and nature of the ribonuclease-resistant, TCA-insoluble residues of the virus nucleic acids. From the composition of these residues and the specificity of ribonuclease, it could be suggested that they average six nucleotides in length. The sedimentation constant which was obtained with the TMV material is consistent with this size. A similar figure was suggested by Volkin and Cohn (15) for certain oligonucleotides remaining after ribonuclease digestion of yeast and rat liver ribonucleic acids. Their oligonucleotides were characterized by low solubility at pH 2 and strong attachment to Dowex resins, whereas ours are defined by their precipitability with 6 per cent TCA. Markham and Smith (8) report a somewhat smaller average size for the ribonuclease-resistant portions of yeast and turnip-yellow mosaic virus nucleic acids, but they defined their resistant fraction on still another basis; namely, as the portion of the digest which failed to pass through a dialysis membrane under certain conditions. This fraction might well differ from that precipitable by 6 per cent TCA.[2]

SUMMARY

The nucleic acids obtained from five strains of tobacco mosaic virus were digested with ribonuclease at 23° for 36 hours at pH 7.6. The ribonuclease-resistant residues which were precipitable by 6 per cent trichloroacetic acid were analyzed for their content of purine and pyrimidine bases. Within the limits of the method of analysis used, the ribonuclease-resistant residues of all five strains appeared to have the same composition. The residues were rich in purine and poor in pyrimidine and averaged about six nucleotides in composition.

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[2] Another difference between the Markham and Smith experiments and ours is the use of a higher temperature for digestion (55°). Incubation of our TMV nucleic acid in 0.1 M borate at pH 7.6 and at 55°, without ribonuclease, left 4.2, 5.5, and 11.9 per cent of the nucleic acid non-precipitable with 6 per cent TCA after 12, 18, and 36 hours, respectively. This unexpected result is being further investigated.

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A SENSITIVE METHOD FOR THE DETERMINATION OF RIBONUCLEIC ACID IN TISSUES AND MICROORGANISMS

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The increasing interest in the composition of nucleoproteins has emphasized the need for more specific methods of assay of constituent nucleic acids. In a previous paper (1) a method was reported for the assay of deoxyribonucleic acid (DNA) in tissues and microorganisms based on the reaction of *p*-nitrophenylhydrazine with deoxyribose following hydrolysis of the biological material with 5 per cent trichloroacetic acid (TCA). The present paper reports a method for ribonucleic acid (RNA) assay with an aliquot of the same TCA hydrolysate.

The most commonly employed method for RNA assay is that of Mejbaum (2), in which orcinol is used to develop a blue-green color with furfural formed from ribose when the latter is heated with prescribed amounts of $FeCl_3$-HCl solution. The results must be corrected for DNA interference (3, 4). In a similar method developed by von Euler and Hahn (4) phloroglucinol is employed. DNA does not interfere, but the method is less sensitive than the orcinol procedure. In a third method, used by Davidson and Waymouth (5), the furfural formed from ribose is extracted with xylene and the xylene extract is allowed to react with aniline acetate to form a pink color. Although DNA does not interfere, the method is complicated by the necessity of removing HCl prior to color formation and the photosensitivity of the color (6). However, the extraction procedure offers certain advantages, namely (1) concentration of the furfural formed and (2) elimination or minimization of interfering substances.

In an effort to combine these advantages with a more efficient chromogenic reagent, a method has been developed in which *p*-bromophenylhydrazine (PBPH) reacts quantitatively in acidified xylene-alcohol solution with furfural formed from the liberated ribose of RNA. As seen below, the specific extinction coefficient of the colored product formed with the proposed reagent is about 1½ times that of the colored product resulting from the Mejbaum orcinol procedure when optical densities are taken at their respective wave-lengths of maximal absorption. The color is stable and reproducible. Experiments performed with several carbohy-

drates show that the proposed method is as specific or more so than the orcinol procedure. The presence of DNA does not interfere with the test.

EXPERIMENTAL

Reagents—

1. Trichloroacetic acid, Merck reagent.

2. Xylene, Mallinckrodt, c.p. Some brands of xylene gave a slightly higher blank which was not improved by using the redistilled solvent. A Caulfield Pipettor (Caulfield Safety Devices, Havertown, Pennsylvania) was used for most pipetting operations.

3. NaCl crystals, Bakers', c.p.

4. Ethyl alcohol-HCl solution. 2 ml. of HCl, c.p. (37 per cent), were added to 100 ml. of 95 per cent ethyl alcohol.

5. *p*-Bromophenylhydrazine hydrochloride, Eastman Kodak Company, Rochester, New York. A 2.5 per cent solution of the hydrochloride in ethyl alcohol-HCl solution was prepared fresh daily. It was found expedient to decolorize the solution with charcoal (Norit A, Fisher Scientific Company, New York). About 1 gm. of charcoal per 30 ml. of volume was adequate.

6. Ribonucleic acid, Nutritional Biochemicals Corporation, Cleveland, Ohio. The material was purified, following the method of Kunitz (7), by reprecipitation from glacial acetic acid. Analysis of the RNA showed N 14.6 per cent, P 9.0 per cent.

Materials Analyzed for RNA—

1. Rat tissues. The livers, lungs, and kidneys from young Sprague-Dawley rats were removed immediately after sacrificing the animals and were frozen at once in dry ice.

2. Yeast. Bakers' yeast (Fleischmann), dried *in vacuo*, was used.

3. Bacteria. *Escherichia coli*, from a 17 hour culture and *Proteus ammoniae*, from 17 and 48 hour cultures grown at 37° on horse meat infusion agar, were harvested in 50 ml. of 0.85 per cent saline. The suspensions were treated with 1 ml. of 37 per cent formaldehyde and after 15 minutes the residues were recovered by centrifugation for 10 minutes at 20,000 × *g* in a Servall SS-1 centrifuge. The residues were washed twice with water, recovered by centrifugation, and dried *in vacuo*.

The biological materials were prepared for analysis by acid washing with cold 10 per cent TCA, defatting with alcohol and ether, and finally converting to a dry powder as described (1). For purposes of testing analytical procedures, the dry material serves as a convenient source of reproducible material from one experiment to another. However, the analytical procedure to be described may be applied to known amounts of homogenized tissue or microorganism following the acid-alcohol-ether extractions, without conversion to powder.

Analytical Procedure

Hydrolysis—In the manner previously described (1), the material to be analyzed was hydrolyzed with 5 per cent TCA for 30 minutes in a boiling water bath followed by dilution with an equal volume of 5 per cent TCA. Since, in some experiments to follow, it was desired to have sufficient aliquots for other assays, 3 ml. of 5 per cent TCA were used for hydrolysis after which an additional 3 ml. volume of 5 per cent TCA was added. In other experiments, hydrolysis was with 2 ml. of 5 per cent TCA followed by an additional 2 ml. volume. 1 ml. of the diluted hydrolysate was equivalent to 0.75 to 6.0 mg. of the dry powder and to 9 to 200 γ of RNA.

100 ml. of a stock solution of standard hydrolyzed RNA (1 ml. = 1 mg.) were prepared in a similar manner. RNA standards of lower concentration were prepared by appropriate dilution of this stock solution with 5 per cent TCA.

Conversion to Furfural—To 1 ml. of the diluted hydrolysate in a 12 ml. centrifuge tube was added 1 ml. of 8 N HCl followed by 1 ml. of xylene and enough NaCl crystals to saturate the mixture. 1 ml. of a standard solution of hydrolyzed RNA of appropriate concentration (usually 150 γ) and 1 ml. of 5 per cent TCA, constituting the blank, were treated likewise. The centrifuge tubes, the mouths of which were covered with sealed ampul bulbs, were placed in a boiling water bath for 3 hours. After cooling, 2 ml. of xylene were added to each tube and the contents were mixed and centrifuged.

Color Development—2 ml. of the xylene layers were transferred to 5 ml. volumetric flasks to which were added 2 ml. of the PBPH reagent. After mixing the contents, the flasks were stoppered with ground glass stoppers and placed in a constant temperature room at 37° for 1 hour. The contents of the flasks were diluted to volume with ethyl alcohol-HCl solution, mixed, and transferred to matched ½ inch Bausch and Lomb tubes. Optical densities of the solutions were measured against the blank by means of the Bausch and Lomb Spectronic 20 colorimeter set at 450 mμ.

Standardization and Validity of Method

Optimal Conditions for Hydrolysis with 5 Per Cent TCA—When the proposed method was applied to 15 and 30 minute hydrolysates, no difference in color intensities of the final solutions was observed. However, in order to use the same hydrolysate for both RNA and DNA determinations, the 30 minute hydrolysis period recommended for DNA (1) was adopted.

Optimal Conditions for Conversion of Ribose to Furfural—Two variables were studied: time of heating in a boiling water bath (1 to 6 hours) and concentration of HCl (1 to 8 N), while the amount of hydrolyzed RNA and the volume of the final mixture were held constant. A 6 N HCl concen-

tration with 3 hours of heating was found to be optimal. Higher concentrations of HCl resulted in appreciable furfural destruction.

Since Foster (8) has shown that the presence of NaCl in the acid conversion medium enhances the production of furfural from pentoses with lower acid concentration, the above experiment was repeated by using final acid concentrations of from 1 to 6 N and saturating each mixture with NaCl. Under these conditions and with 3 hours of heating the results with a 4 N HCl concentration were about 10 per cent higher than that with a 6 N HCl concentration without NaCl. Furthermore, the time of heating was not as critical as without NaCl.

Optimal Conditions for Color Formation—The amount of PBPH reagent prescribed was found sufficient for the assay of at least 210 γ of standard RNA. Doubling the amount of reagent at this concentration gave no further increase in color intensity. Color development took place slowly at room temperature, and a maximum was attained sooner (60 minutes) at 37° in an incubator room (or a slightly shorter time in a constant temperature bath at this temperature). The color was stable for at least 2 hours.

Although HCl was necessary to the reaction and to stabilize the reagent, its concentration was not critical. Doubling the concentration gave no increase in color intensity nor shortened the period for color development. Larger volumes of HCl, however, were insoluble in the xylene-alcohol mixture.

Source of Reactive Ribose—Purified yeast RNA (N 14.9 per cent, P 9.0 per cent) was analyzed by the proposed method with recrystallized D-ribose as a standard. The RNA was found to yield 22.2 per cent ribose, which was about 50 per cent of the total theoretical ribose content of RNA as calculated from the tetranucleotide formula. Since yeast RNA composition has been found to correspond approximately to the tetranucleotide formula, and since purine nucleotides hydrolyze easily while pyrimidine nucleotides do not, it is likely that the purine nucleotides were the main source of reactive ribose. To demonstrate further the probability of this assumption, the purity of the RNA can be calculated, based on the theoretical amount of purine nucleotide ribose (23.4 per cent calculated from the tetranucleotide formula). This purity was computed to be 94.9 per cent and corresponds well with that calculated from nitrogen analysis (91.4 per cent) or from phosphorus analysis (94.7 per cent).

Standardization of Test—To show that the amount of colored product formed with PBPH is directly proportional to the amount of RNA present and to determine the reproducibility of the procedure, the method was applied to various dilutions of hydrolyzed RNA standard solution. One set of such dilutions, along with a reagent blank, was run independently

eight times and optical density readings were made against distilled water. The resulting direct proportionality is shown in Fig. 1.

The reproducibility for the eight determinations at each concentration level may be shown by the relative mean deviations. At concentrations of 9.4, 18.8, 37.5, 75.0, and 150 γ the mean deviations, expressed as a per cent of their respective means, were 7.4, 4.0, 3.9, 4.5, and 4.5, respectively.

Spectral Characteristics—Spectral transmittance curves presented in Fig. 2 show the absorption characteristics of the colored product resulting from

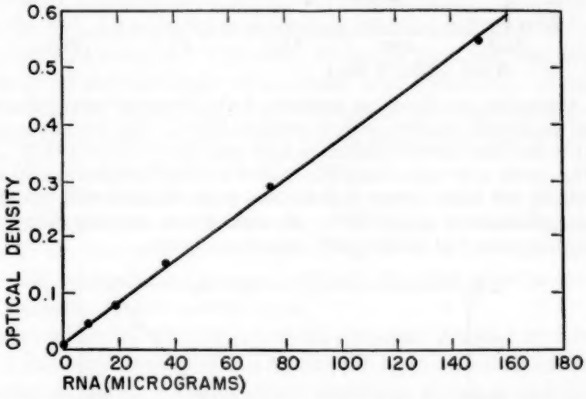

FIG. 1. Standardization curve, showing the relationship between optical density and amount of RNA, as obtained by the *p*-bromophenylhydrazine method. The amounts of RNA represent the initial amounts of hydrolyzed RNA taken. The final aliquots used for color development contained the equivalent of two-thirds of these initial amounts, as described in the assay procedure. Each point represents the mean of eight determinations. The readings were made against distilled water with matched ½ inch tubes in the Bausch and Lomb Spectronic 20 colorimeter at 450 mμ.

the PBPH reaction. For comparison, the curve for the colored product resulting from the orcinol reaction is also given. These curves represent solutions of the same concentration of RNA (15 γ per ml.) read against their respective blanks and illustrate the quantitative sensitivity of the two reactions. The extinction coefficients at their respective maxima (450 mμ for the PBPH product and 660 mμ for the orcinol product) show the absorption of the former to be about 1½ times that of the latter.

Relationship between Amounts of Tissue Powder and Amounts of RNA Found—Fig. 3 illustrates the results of an experiment in which the method was applied to increasing amounts of liver tissue powder. It will be seen that there is a direct proportionality between optical density readings and the amounts of powder used.

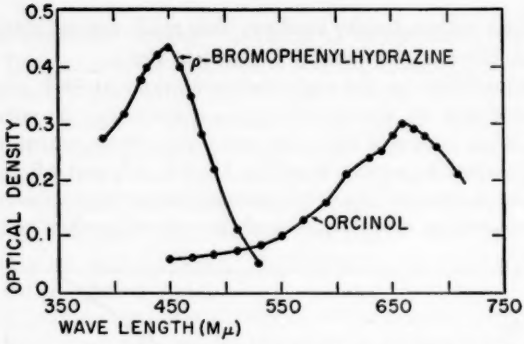

FIG. 2. Absorption spectra of the products of the *p*-bromophenylhydrazine reaction and of the orcinol reaction with hydrolyzed RNA. The initial concentration was such that the final colored solution in each case was equivalent to 15 γ of RNA per ml. The curves were taken from the Bausch and Lomb Spectronic 20 colorimeter. Essentially the same curves and maxima were obtained with the Beckman quartz spectrophotometer (model DU). Matched ½ inch test-tube cells were used, and the solutions were read against their respective blanks.

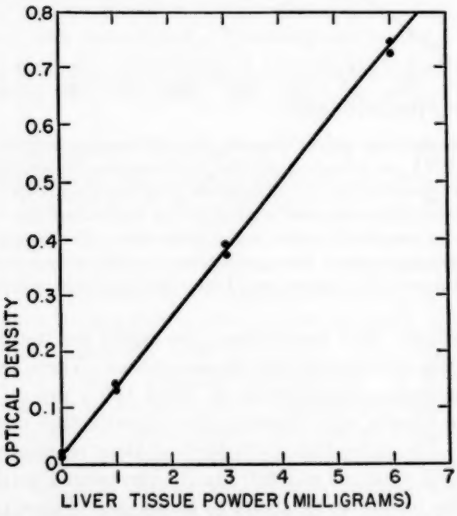

FIG. 3. Application of the *p*-bromophenylhydrazine method to increasing amounts of liver tissue powder. Each amount of tissue powder represents the initial amounts of hydrolyzed liver powder per ml. The two determinations at each level were made on separate hydrolysates. As in Fig. 1, the final aliquots used for color development contained the equivalent of two-thirds of the hydrolyzed RNA that was in the weight of tissue powder shown. The optical density readings were made as described in Fig. 1.

Recovery of RNA—A given volume of hydrolyzed RNA standard solution was added to a given volume of hydrolysate of liver tissue powder so that a 1 ml. volume of the mixture was equivalent to 1 mg. of liver tissue powder and to a known amount of RNA. Three concentrations of hydrolyzed RNA were used so that the final equivalences of added RNA, per ml., were 20, 40, and 80 γ. After applying the test procedure, the average of duplicate determinations made at each level showed recoveries ranging from 98 to 100 per cent.

Specificity—The compound produced from ribose and RNA, under conditions of the test, gave the same absorption maxima and spectrophotometric curves with PBPH as pure furfural. It should be noted that some carbohydrates yield this aldehyde with similar acid treatment. However, the proposed method, in contrast to the orcinol or phloroglucinol procedures, eliminates those other possible interfering substances which do not yield furfural and are not extractable by xylene.

Under conditions of the proposed test, the assay of 1 mg. of deoxyribose anilide[1] gave no color, and from 1 mg. of DNA only a trace was observed. This small amount of color presumably came from an impurity, possibly RNA itself, inasmuch as present methods of preparation may not completely separate the two nucleic acids.

Galacturonic acid, which gave about the same amount of color as that given by the same weight of RNA, was the most serious possible interfering substance tested. Among other materials, glycogen and methyl glucoside yielded about one-twentieth, while levulose, dextrose, and saccharose yielded one-tenth of the color given by the same weight of RNA. Though these interfering substances are soluble in one or both extracting solvents used in preparing the biological material for analysis, there is no assurance of their complete absence. For example, some furfural-yielding materials (polysaccharides containing uronic acid groups) are bound to proteins in the native state (9). It is conceivable that they would not be removed by the cold extractions but would be released, at least in part, by heating with 5 per cent TCA.

Application of Method and Comparison of Results

In order to determine the applicability of the proposed method, the results with PBPH were compared with those with orcinol and with phosphorus and ultraviolet absorption measurements in the following manner. The RNA content of extracted powders of several tissues and microorganisms was determined by the PBPH and orcinol methods (2) and the corresponding phosphorus and ultaviolet absorption values were calculated. Similarly, phosphorus and ultraviolet absorption values were calculated

[1] Deoxyribose anilide may be obtained from the California Foundation for Biochemical Research, Los Angeles, California.

from results of DNA assays (1). By employing the RNA phosphorus results as found by the two RNA methods, two figures for total phosphorus content were calculated by adding to each RNA phosphorus value the DNA phosphorus content. These calculated results were then compared with total phosphorus content found by direct determination.[2] The calculated phosphorus contents were based on the average phosphorus contents found experimentally for the two nucleic acids used for standards, 9.0 per cent for RNA and 7.9 per cent for DNA.[3] Calculated ultraviolet absorption values were summed in a similar manner and compared with ultraviolet absorption measurements found directly. The ultraviolet values were calculated from extinction coefficients ($E_{1\text{ cm.}}$) of 0.224 and 0.285 found for 0.001 per cent solutions of hydrolyzed DNA and RNA standards, respectively, as determined at 260 mμ in the Beckman quartz spectrophotometer (11). The same hydrolysate was used for the different assays and quadruplicate analyses were performed on each material.

The data obtained are summarized in Tables I, II, and III.

Analyses of Tissues and Microorganisms for RNA by PBPH and Orcinol Methods and for DNA by p-Nitrophenylhydrazine Procedure—The results obtained are reported in Table I. It may be seen that, with the exception of yeast, the RNA values as found by the PBPH method, although somewhat lower, were in fair agreement with RNA values as found by the orcinol procedure. The RNA values for yeast by the latter method were obviously increased by a brownish red non-specific color which contributed to the total absorption and for which no adequate correction could be made. Levy *et al.* (12), using the phloroglucinol procedure of von Euler and Hahn (4), experienced similar difficulty with yeast. Perhaps the consistently higher RNA values found here for the other materials by the orcinol method could also be attributed to such non-specific color.

The orcinol RNA values shown have been corrected for DNA interference. Experimentally, DNA gave 12.5 per cent of the color given by the same weight of RNA at 660 mμ. This was the same amount of correction found necessary by von Euler and Hahn (4).

The reproducibility of the PBPH method is illustrated by the data

[2] Phosphorus determinations were performed by the molybdenum blue reaction. The reducing agent was 0.5 per cent *p*-hydroxyphenylglycine in 2.5 per cent Na_2SO_3 solution. After dilution of the H_2SO_4 digests with 10 ml. of H_2O, 2 ml. of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (2.5 per cent in 9 N H_2SO_4), followed by 1 ml. of the reducing agent, were added. A stable color was developed in an approximately 2 N acid concentration by heating for 30 minutes in a boiling water bath. After dilution to 15 ml., the solutions were read at 660 mμ.

[3] The DNA, prepared from calf thymus by the method of Hammarsten (10), also gave an analysis of 13.7 per cent nitrogen.

TABLE I

Analyses of Tissues and Microorganisms for RNA by p-Bromophenylhydrazine and Orcinol Methods and DNA by p-Nitrophenylhydrazine

Original material	Analysis No.	RNA, γ per mg. extracted powder		DNA, γ per mg. extracted powder
		PBPH	Orcinol*	
Rat liver	1	36.9	37.9	13.4
	2	34.7	38.5	13.5
	3	32.0	37.8	12.3
	4	34.2	39.4	12.3
Average		34.5	38.4	12.9
Rat lung	1	15.5	16.1	37.8
	2	13.3	12.9	40.4
	3	13.1	14.6	39.9
	4	15.1	17.3	38.0
Average		14.3	15.2	39.0
Rat kidney	1	22.0	28.1	20.6
	2	20.7	24.5	21.8
	3	20.4	24.7	20.6
	4	20.8	24.8	20.2
Average		21.0	25.5	20.8
Yeast	1	75.0	124.4	5.1
	2	72.5	104.6	5.0
	3	66.3	113.7	4.5
	4	69.5	124.4	4.8
Average		70.8	116.8	4.9
E. coli	1	127.5	132.1	58.8
	2	127.5	128.2	58.2
	3	108.5	122.3	52.0
	4	125.9	129.4	53.9
Average		122.4	128.0	55.7

* Corrected for DNA interference, but not for other non-specific color (see the text).

TABLE II

Total Nucleic Acid Phosphorus Content Found in Tissues and Microorganisms and That Calculated from RNA and DNA Methods

DNAP = deoxyribonucleic acid phosphorus; RNAP = ribonucleic acid phosphorus.

Original material	Analysis No.	Phosphorus, γ per mg. extracted powder		
		Found	Calculated (see text)	
			DNAP + RNAP (PBPH)	DNAP + RNAP (orcinol)
Rat liver	1	4.3	4.4	4.5
	2	4.5	4.2	4.5
	3	4.3	3.9	4.4
	4	4.3	4.1	4.5
Average		4.4	4.2	4.5
Rat lung	1	4.4	4.4	4.4
	2	4.7	4.4	4.4
	3	4.5	4.3	4.5
	4	4.4	4.4	4.6
Average		4.5	4.4	4.5
Rat kidney	1	3.7	3.6	4.2
	2	3.7	3.6	3.9
	3	3.8	3.5	3.9
	4	4.0	3.5	3.8
Average		3.8	3.6	4.0
Yeast	1	7.2	7.2	11.6
	2	7.3	6.9	9.8
	3	7.3	6.3	10.6
	4	7.1	6.6	11.6
Average		7.2	6.8	10.9
E. coli	1	14.9	16.1	16.5
	2	15.2	16.1	16.1
	3	14.6	13.9	15.1
	4	14.9	15.6	15.9
Average		14.9	15.4	15.9

TABLE III

Total Nucleic Acid Ultraviolet Absorption Found for Tissues and Microorganisms and That Calculated from RNA and DNA Methods

Original material	Analysis No.	Optical density* per 0.1 mg. extracted powder per ml. TCA		
		Found	Calculated (see text)	
			DNAP + RNAP (PBPH)	DNAP + RNAP (orcinol)
Rat liver	1	0.126	0.135	0.138
	2	0.130	0.129	0.140
	3	0.129	0.119	0.135
	4	0.128	0.125	0.140
Average		0.128	0.127	0.138
Rat lung	1	0.126	0.129	0.131
	2	0.120	0.128	0.127
	3	0.125	0.127	0.131
	4	0.123	0.128	0.134
Average		0.124	0.128	0.131
Rat kidney	1	0.116	0.109	0.126
	2	0.123	0.108	0.119
	3	0.113	0.104	0.117
	4	0.112	0.105	0.116
Average		0.116	0.107	0.120
Yeast	1	0.188	0.225	0.366
	2	.0.188	0.218	0.309
	3	0.180	0.199	0.334
	4	0.179	0.209	0.365
Average		0.184	0.213	0.344
E. coli	1	0.453	0.495	0.508
	2	0.403	0.494	0.495
	3	0.384	0.426	0.465
	4	0.392	0.480	0.490
Average		0.408	0.474	0.490

* 1 cm. light path at 260 mμ.

shown. With the five different materials on which four analyses are reported on each, the per cent deviation of each determination from its respective mean of four such determinations can be calculated. By the

PBPH method the average of all twenty of the per cent deviations was 4.7 and the maximal single per cent deviation was about 11 per cent. By the orcinol method, the average of the deviations (omitting the yeast determinations) was about the same (4.6), whereas the maximal single deviation was about 15 per cent.

Comparison of Total Phosphorus and Ultraviolet Absorption Values Found Experimentally with Calculated Data from Nucleic Acid Assays—The results obtained are given in Tables II and III. It may be seen that calculated data for yeast, based on the RNA results as determined by the orcinol method, reflected the extraneous absorption, since significantly higher phosphorus and ultraviolet absorption values than those found by actual assay were obtained. On the contrary, calculated data for this material, based on the RNA results found by the PBPH method, agreed satisfactorily with values obtained by actual assay. For rat tissues, it is obvious that there was good agreement between phosphorus and ultraviolet absorption values calculated and those found, regardless of which RNA value was taken. For *E. coli*, however, calculated data were in greater agreement with those of actual assays when RNA values as found by the PBPH reaction were taken.

TABLE IV

Conversion of Nucleic Acid Values Found for Extracted Powders to Corresponding Values for Original Biological Material

Tissue or organism	Average RNA, γ per mg. extracted powder		Average DNA, γ per mg. extracted powder	Mg. extracted powder per 100 mg. original material†	Average RNA, mg. per 100 mg. original material		Average DNA, mg. per 100 mg. original material
	PBPH	Orcinol*			PBPH	Orcinol*	
Rat liver	34.5	38.4	12.9	17.2	0.59	0.66	0.22
" lung	14.3	15.2	39.0	13.3	0.19	0.20	0.52
" kidney	21.0	25.5	20.8	14.0	0.29	0.36	0.29
Yeast	70.8	116.8	4.9	73.2	5.18	8.55	0.36
E. coli	122.4	128.0	55.7	69.2	8.54	8.86	3.85

* See foot-note to Table I.

† Wet weight for rat tissues; dry weight for yeast and *E. coli*.

There was a tendency, in general, for greater agreement between calculated and found phosphorus values than between calculated and found ultraviolet absorption values, regardless of which RNA results were involved.

Conversion of Nucleic Acid Values of Extracted Powders to Equivalent Values for Original Material—For comparison with data in the literature,

which are often given as per cent of wet weight in the case of animal tissues, or per cent of dry weight in the case of microorganisms, the nucleic acid values for the extracted powders (Table I) have been converted to corresponding values for the original material. Table IV gives the values obtained for the several tissues and microorganisms when their average RNA values, as found by the two methods, and their average DNA values are multiplied by their respective conversion factors (mg. of extracted powder per 100 mg. of original material).

DISCUSSION

Since only the purine nucleotides hydrolyze readily to give pentose, in the PBPH procedure, as in the orcinol method, essentially only purine-bound ribose is measured. Hence, RNA determination based on the carbohydrate moiety is complicated by possible differences in the purine-pyrimidine nucleotide ratio from one nucleic acid to another. That considerable variations in the purine-pyrimidine ratio can occur has been demonstrated by Chargaff *et al.* (13–15) and also Loring (16) who found the purine content of tobacco mosaic virus to be lower by 20 per cent than that required by the tetranucleotide hypothesis. DNA does not present this difficulty, as its purine-pyrimidine ratio has been shown to be relatively constant (13, 17).

In the present investigations, differences between calculated and found phosphorus values or calculated and found ultraviolet absorption values for the several materials analyzed could indicate differences in RNA composition as compared to the standard. The case of *P. ammoniae*, studied in a similar manner at two stages of growth (17 and 48 hours), offers a more conclusive illustration of this point; for both phosphorus and ultraviolet calculated values were significantly higher than found values, regardless of which RNA results were taken in the calculations. It is of interest that less variation between calculated and found values was observed in the 48 hour culture of this organism than in the 17 hour culture. Since by both the orcinol and PBPH methods, the amount of RNA in the 17 hour culture was about twice that in the 48 hour culture, whereas the DNA content was approximately the same in both, one implication may be that, with increasing amounts of RNA, differences in RNA composition as compared to the yeast RNA standard become more apparent.

Besides compositional and structural differences from one nucleic acid to another, there may be present interfering substances which give a positive test with both the orcinol and PBPH reagents. Thus, it is well to check nucleic acid results found by colorimetric sugar methods against to-

tal phosphorus and ultraviolet measurements. However, possible structural differences in the polynucleotide group, as suggested by Gulland (18), or small amounts of non-nucleic acid phosphorus could cause erroneous conclusions from phosphorus determinations. Likewise, besides variations in the proportion of different bases, ultraviolet measurements may be affected by the presence of other materials which absorb in the ultraviolet for which no adequate correction can readily be made.

It should be noted that the proposed method, by virtue of the extraction before color formation, can eliminate or minimize effects of interfering substances or non-specific color resulting from organic matter charred by strong acid. For example, in preliminary experiments with formalin-fixed tissue which gave non-specific color with both the orcinol and phloroglucinol methods, the PBPH method yielded results which were suggestive of its usefulness for the analysis of such material.

SUMMARY

A colorimetric procedure has been devised for determining the ribonucleic acid (RNA) content of biological material. Ribose, from hydrolyzed RNA, when heated in a 4 N HCl medium saturated with NaCl, yields furfural which is extractable with xylene. The xylene extract reacts with *p*-bromophenylhydrazine (PBPH) in HCl-ethanol solution to give a yellow color (maximal absorption at 450 mμ) which, under standardized conditions, is a measure of the amount of RNA present. The developed color followed Beer's law over the range of 9.4 γ to more than 150 γ of purified RNA. The PBPH method was found to be more specific than the orcinol procedure, the one most generally used for RNA assay, since deoxyribonucleic acid (DNA) did not interfere with the test and the xylene extraction minimized the effect of interfering substances. The results were reproducible and good recoveries were obtained.

RNA values for several tissues and microorganisms were determined by the proposed method and the orcinol procedure, and thoeretical phosphorus content and ultraviolet absorption were calculated from each result. Total phosphorus content, calculated by adding RNA phosphorus values to the phosphorus content calculated from deoxyribonucleic acid assays on the same materials, was compared with total phosphorus values found by actual assay. Calculated ultraviolet absorption values were treated likewise. In general, the calculated phosphorus and ultraviolet values based on PBPH assays for RNA agreed more closely with experimental findings than did those based on the orcinol assays. Furthermore, such comparisons indicated that the proposed method may be applicable to more diverse materials.

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ENZYMIC SYNTHESIS OF A NEW DINUCLEOTIDE FROM COZYMASE BY A NEW METHOD OF BIOSYNTHESIS

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Recently a novel mechanism of biosynthesis has been uncovered (1) which seems to be energized from "onium" salts. In reactions of this class a quaternary ammonium salt (*e.g.* thiamine) or a sulfonium salt (*e.g.* adenosylmethionine) is activated by a specific enzyme and transfers an alkyl group to an acceptor molecule. A shared electron in the "onium" linkage becomes unshared in the reaction products, and a hydrogen ion is formed. Because of this partial transfer of an electron, one can picture the reaction as a reduction.

Only two members of this class of reactions have been thus far recognized, *viz.* the thiaminase-catalyzed syntheses and those brought about by the methyl transferases which act on adenosylmethionine (2). Consequently, it would seem desirable to search for other examples. Because many kinds of quaternary ammonium compounds are known to occur in living things, the idea seemed justifiable (1) that there might be many examples of this class of reaction. Thus, it was suggested that cozymase (DPN),[1] one of these quaternary ammonium compounds, might undergo such a reaction with the consequent production of a new dinucleotide. A short note has appeared (3) which describes this reaction in which the acceptor was 4-amino-5-carboxamidoimidazole, a recognized precursor of the purines in microorganisms and animals. The purpose of this paper is to give a detailed account of this work.

The enzyme catalyzing this reaction was beef spleen DPNase. The product of the reaction was isolated in apparently pure form, and was shown to be a pyrophosphate of the imidazole ribotide and of adenylic

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[1] Throughout this paper the following abbreviations have been used: DPN, diphosphopyridine nucleotide; IRPA, the pyrophosphate derived from the 4-amino-5-carboxamidoimidazole ribotide (IRP) and AMP (the initials stand for imidazoleribose-phosphate-adenylic acid); RPA, ribose-phosphate-adenylic acid; IRP, 3-riboside-5′-phosphate-4-amino-5-carboxamidoimidazole (the initials stand for imidazoleribose-phosphate); AMP, adenosine-5′-phosphate; NRP, nicotinamide mononucleotide; the imidazole, 4-amino-5-carboxamidoimidazole; DPNase, the enzyme hydrolyzing the bond between nicotinamide and ribose in the oxidized form of DPN; Tris, tris(hydroxymethyl)aminomethane.

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acid. The name of this compound was abbreviated to IRPA, and the course of the reaction and the structure of the dinucleotide are outlined in Fig. 1.

This reaction is not the same as the base-exchanges of DPN which have been studied by Zatman *et al.* (4, 5). At least three features which distinguish the two types have been noted.

The base-exchange reactions involve no loss of the energy-rich "onium" linkage. Thus, the exchanges have been found to proceed between DPN and isonicotinic acid hydrazide, β-acetylpyridine, or other pyridine derivatives. The nicotinamide portion of DPN is exchanged for the new pyridine base, which is held as a pyridinium salt, just as the nicotinamide was

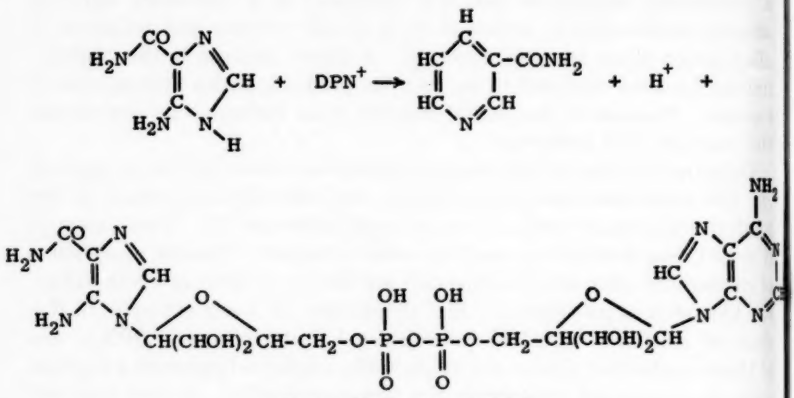

FIG. 1. Formation of IRPA

in DPN. No loss of the quaternary linkage has occurred. On the contrary, in the new reaction the product is no longer a quaternary ammonium salt. The "onium" linkage has been lost.

In the base-exchange reactions, as a consequence of retention of the "onium" linkage, no H^+ is liberated. By contrast, the new reaction involves formation of an H^+.

The base-exchange reactions are readily reversible. On the contrary, the new reaction is irreversible, and the new dinucleotide formed is not attacked by DPNase. This irreversibility of the new reaction is exactly analogous to the situation with thiaminase and the methyl transferases, which do not readily yield back from the products any of the starting materials (1).

EXPERIMENTAL

Materials and Methods—Soluble and purified beef spleen DPNase was prepared according to the method of Alivisatos and Woolley (6). The

DPN used was mainly from the Sigma Chemical Company, 70 per cent purity, but crucial experiments were repeated, either with Sigma 90 per cent DPN or with samples purified in this laboratory by electrophoresis. DPN was determined either by conversion to the cyano derivative (7) or by means of alcohol dehydrogenase (4). The 4-amino-5-carboxamidoimidazole was made by the method of Shaw and Woolley (8), either in this laboratory or at the California Foundation for Biochemical Research. All enzymic reactions were buffered with Tris (tris(hydroxymethyl)aminomethane). Paper chromatography was carried out by the ascending method with Whatman No. 1 paper, and the positions of the spots were determined in ultraviolet light from a Mineralite lamp. Spots of diazotizable compounds were located by colors developed with the Bratton-Marshall method.[2] Paper electrophoresis was carried out according to the method of Kunkel and Tiselius (9). All the values for mobilities were determined in reference to DPN, which was run alongside. Dowex 1, chloride form, columns were used essentially as described by Cohn and Carter (10). Diazotizable amines were determined according to the method of Bratton and Marshall (11). Because different modifications of this procedure are now in common use and were found not to give identical results, the exact conditions used in this work must be described. The sample (2.4 ml. in water) was treated with 0.15 ml. of 79 per cent trichloroacetic acid and 0.15 ml. of 0.1 per cent aqueous sodium nitrite. 3 minutes later 0.15 ml. of ammonium sulfamate (0.5 per cent) was added and 2 minutes later 0.15 ml. of 0.1 per cent *N*-(1-naphthyl)ethylenediamine dihydrochloride. The color was measured 10 minutes later with a Beckman model DU spectrophotometer.

Formation of New Dinucleotide (IRPA)—1 gm. of 4-amino-5-carboxamidoimidazole hydrochloride (6135 μmoles) and 0.264 gm. of 70 per cent pure DPN (272 μmoles) were dissolved in 5 ml. of 1 M Tris, pH 7.5. Enough KOH and water were added to bring the pH back to 7.45 and the final volume to 11 ml. Purified DPNase (330 units in 0.5 ml.) was added, and the mixture was incubated at 38° for 19 hours. 88.5 per cent of the DPN originally present had then disappeared (alcohol dehydrogenase method).

Isolation of IRPA by Chromatography—The reaction mixture described above was diluted to 1100 ml. and passed through a Dowex 1 (10 per cent cross-linked) chloride column 1 × 10 cm. which had been equilibrated with

[2] To avoid spreading of the spots which otherwise occurred after spraying with the four Bratton-Marshall reagents, the following modification proved useful. The paper was sprayed first with a solution made from 1 volume of 0.1 per cent aqueous $NaNO_2$ cooled to 0° and slowly treated with 1 volume of 20 per cent trichloroacetic acid. After 3 minutes the paper was sprayed with the 0.1 per cent ethylenediamine reagent.

distilled water. The flow rate was 20 ml. per hour. After the reaction mixture had passed through the column, the latter was washed with water, until the effluent was free of diazotizable amines (about 400 ml.). The solvent then was changed to 0.0045 N HCl, pH 2.35, and the washing was continued until the effluent was free of ultraviolet-absorbing material (470 ml.). The solvent was then changed to 0.0050 N HCl, pH 2.31, and the first 100 ml. of effluent were discarded. The point of emergence of the dinucleotide was judged from the absorption at 260 mμ and from the test for diazotizable amines.[3] The fraction from 100 to 540 ml. contained the pure dinucleotide (IRPA). The column was then washed with the same solvent until the effluent was free of diazotizable amine or ultraviolet-absorbing material, and this fraction was recycled with subsequent runs. The nicotinamide-free hydrolytic product of DPN (*i.e.* RPA) remained on the column and could be obtained by further elution with 0.0055 N HCl, pH 2.26 (12).

The effluent containing the IRPA (440 ml.) was mixed with 4 liters of ethanol and 4 liters of ether at 4°. The precipitate was allowed to settle in a tall cylinder at −16°. Most of the supernatant fluid was then decanted, and the precipitate was collected on a fine fritted glass filter and washed with ether. The precipitate was dissolved in 1.5 ml. of water, the solution was filtered,[4] and the IRPA was reprecipitated with ethanol and ether, dried over P_2O_5 at 25° *in vacuo*. The final yield was 40 mg. of purplish white powder.

Isolation of IRPA by Paper Electrophoresis—The method of Kunkel and Tiselius (9) was used. The buffer was 0.05 M ammonium acetate, pH 5.0, and the potential difference across the ends of the papers was 200 volts for 20 hours at 4°. The paper was Whatman No. 1, 18 × 4.5 inches, and the starting line was 7 inches from the cathode end. Thirty such sheets were run as a pile at one time. After the run, one of the papers was cut into sections, eluted with water, and the position of the IRPA determined by the ultraviolet absorption and Bratton-Marshall tests on the eluates. This was used as a guide for cutting the other papers. The proper eluate from the first run was freeze-dried and rerun on a single sheet at 600 volts with 0.1 M ammonium acetate, pH 5.0, for 4 hours. The concentration of dinucleotide applied was 3 μmoles per ml. The relative positions occupied by DPN, IRPA, and other pertinent compounds are given in Fig. 2. In a single run each of these compounds could be clearly separated from the

[3] This was after the first 40 ml., but to obtain pure material it was best to begin collecting after 100 ml.

[4] A dark insoluble oxidation product was thus removed. Formation of this dark material was very troublesome and occurred excessively when the effluent was concentrated by freeze drying. For this reason it was best to precipitate directly with alcohol and ether.

others. The IRPA migrated 2.2 times as fast as DPN. These two dinucleotides differ in that DPN contains a strongly basic "onium" linkage which is absent in IRPA.

Factors Influencing Yield of IRPA—The yield of IRPA depended primarily on the concentration of 4-amino-5-carboxamidoimidazole at the beginning of the reaction. This is shown by the data in Table I. The yield was calculated on the basis of the amount of DPN which disappeared

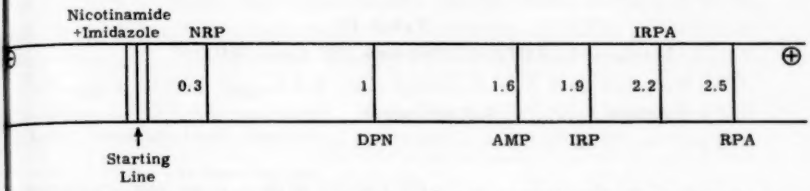

FIG. 2. Relative positions in paper electrophoresis of IRPA, IRP, DPN, and other nucleotides. The conditions were 0.1 M ammonium acetate, pH 5.0, 600 volts, 4 hours, 4°. The paper was 46 cm. long, the actual distance moved by DPN was 6.5 cm.

TABLE I

Dependence of Yield of IRPA on Concentration of 4-Amino-5-carboxamidoimidazole

Imidazole	Initial DPN	Utilized DPN	Enzyme	Period of incubation	Yield based on DPN used
μmoles per ml.	*μmoles per ml.*	*μmoles per ml.*	*units per ml.*	*hrs.*	*per cent*
6	4.16	2.91	2.65	24	0.5
74	4.44	3.63	6.60	19	9.0
148	5.32	5.00	6.60	24	19.0
296	4.55	4.38	8.25	3	27.2
296	5.32	4.45	6.60	18	29.7
396	4.77	4.76	27.20	24	34.8
575	23.70	19.65	55.50	19	36.7

The reaction mixture was buffered, in all cases, with Tris, pH 7.4, 0.1 to 0.4 M (final concentration). DPN was determined at zero time and at the end of the incubation (alcohol dehydrogenase).

from the reaction mixture. The IRPA was separated by electrophoresis or by column chromatography, and the total amount was measured by the Bratton-Marshall method.

The yield of the dinucleotide did not depend on the concentration of the enzyme. When the enzyme was reduced in amount, the longer incubation period required for the disappearance of DPN still resulted in the same yield (fourth and fifth lines, Table I).

The absolute amount of IRPA formed was increased by increasing the concentration of DPN, but the percentage yield, based on the DPN which

disappeared, was independent of the DPN concentration. The nicotinamide set free during the reaction caused some inhibition of the enzyme, especially when the DPN concentration was large. For this reason, the time of incubation had to be extended. Nevertheless, the percentage yield was unaffected. Clearly, the imidazole competed with water in the reaction. The more imidazole present with respect to water, the greater was the yield of IRPA.

TABLE II

Analytical Data from IRPA and IRP

Constituent	Analytical method*	IRPA		IRP	
		Calculated	Found	Calculated	Found
		moles per 667 gm.	*moles per 667 gm.*	*mole per mole ribose*	*mole per mole ribose†*
Ribose	(13)	2.00	2.03	1.00	1.00
Total P	(14, 15)	2.00	1.93	1.00	0.94
Inorganic P		0.00	0.00	0.00	0.00
" " after pyrophosphatase + phosphatase		2.00	1.80	1.00	
Imidazole	(11)‡	1.00	1.00	1.00	0.95
Adenine	Ultraviolet at 260 mμ§	1.00	0.96	0.00	
AMP	Isolation after pyrophosphatase‖	1.00	0.95	0.00	
IRP	" "	1.00	0.96	1.00	

* The numbers in parentheses refer to references in the literature.

† The solution was analyzed for ribose, and other values were referred to this.

‡ The values from the Bratton-Marshall method were corrected for decreased absorption of 3-substituted imidazoles as described in a succeeding section.

§ Absorption due to imidazole portion of the molecule was subtracted from total absorption at 260 mμ.

‖ The method is described in detail in a succeeding section.

Analysis of IRPA—Weighed samples of IRPA prepared by the chromatographic procedure were used to obtain the data listed in Table II. The methods of analysis are indicated there.

Paper Chromatography of IRPA—In a variety of solvent systems IRPA moved as a single spot at the rates indicated in Table III. The R_F values were the same whether the spot was detected with a Mineralite lamp or with the Bratton-Marshall test.[2, 5] In the solvents tested, DPN could not be completely separated from IRPA.

[5] IRPA gave a purplish blue spot with this test, whereas the free imidazole gave a red spot.

Proof of Structure of IRPA—In addition to the analytical findings the following experiments indicated that the structure of IRPA was that shown in Fig. 1.

Enzymic Cleavage of IRPA to AMP and IRP; Preparation of IRP—6.45 μmoles of IRPA (4.4 mg.) in 4.7 ml. of 0.1 M Tris at pH 7.5 and an excess of nucleotide-pyrophosphatase of Kornberg and Pricer (20)[6] were incubated together for 3 hours at 38°. The products were then separated on a Dowex 1 (10 per cent cross-linked), chloride form, column, 1 × 10 cm., in the manner described earlier for preparation of IRPA. Two peaks were obtained. The first was eluted by 0.002 N HCl and consisted of pure AMP. The second was eluted with 0.0025 N HCl and was pure IRP. No IRPA or RPA was recovered. According to ultraviolet absorption and Bratton-Marshall determinations the AMP amounted to 6.1 μmoles and the IRP to 6.18 μmoles. The two solutions were dried from the frozen state.

TABLE III

R_F Values from Paper Chromatograms Run at 25°

System	IRPA	DPN	RPA	Imidazole	Nicotinamide	AMP	IRP
n-Propanol-water 4:1 (16)	0.00	0.00	0.04	0.40	0.69	0.07	0.08
n-Propanol-diluted NH_4OH 3:2 (16)	0.59		0.54	0.67	0.89	0.56	0.52
n-Butanoldiethylene glycol-diluted NH_4OH (16, 17)	0.00	0.00	0.08	0.54		0.00	0.00
Ethanol-0.1 M acetic acid 1:1 (4)	0.42	0.47	0.66	0.62	0.76	0.58	0.62
K_2HPO_4-isoamyl alcohol (18)	0.67	0.72	0.78	0.60	0.71	0.68	0.80
Isopropanol-2 N HCl (19)	0.15	0.12	0.55	0.53	0.44	0.45	0.50

The numbers in parentheses refer to the literature.

The AMP was identified by comparison with an authentic sample in paper chromatography and electrophoresis (see Fig. 2 and Table III).

R_F values for the IRP are shown in Table III. As noted earlier by Greenberg (21), spots containing this mononucleotide turned purplish blue on standing without any spray.

Structure of IRP—The compound was characterized further by the analyses in Table II and by identification of the products of acid hydrolysis. When heated at 100° for 1 hour in 2 N HCl, this substance yielded 4-amino-5-carboxamidoimidazole, which was identified by paper chromatography, and ribose-5-phosphate, which was identified with an authentic specimen in paper electrophoresis. The positions of the spots of the ribose phosphate were found by the method of Mukherjee and Srivastava (22).

[6] We wish to thank Dr. A. Kornberg for the purified nucleotide pyrophosphatase.

Paper electrophoresis of IRP demonstrated clearly that it was not an "onium" salt (see Fig. 2). The similarly constituted "onium" compound, *viz.* NRP, moved much more slowly than did IRP. One would expect the strongly basic "onium" linkage to retard the migration due to the organic phosphoric acid. The more rapid migration of IRP thus indicated that it was not an "onium" compound. The NRP was prepared by the action of nucleotide pyrophosphatase on DPN (20).

The position of the phosphate ester in the ribotide (IRP) was established by the positive periodate test for 1,2-glycols (23). One could argue that the phosphate group could have migrated to position 3′. If this had been so, then the mononucleotide should have been unattacked by periodate.

Hydrolysis of IRPA with Combination of Enzymes—During the cleavage of IRPA by nucleotide pyrophosphatase no inorganic phosphate was liberated. However, when this reaction mixture was incubated with a semipurified phosphatase from yeast,[7] practically all of the phosphorus appeared as inorganic phosphate (see Table II). The yeast phosphatase by itself liberated from IRPA only 5 per cent of the total phosphorus as inorganic phosphate in the same period of time (15 minutes).

Ultraviolet Absorption Spectra—The absorption spectra of IRPA and of IRP at pH 2.6 and 7.5 are given in Figs. 3, 4, and 5. It is noteworthy that the curves obtained by adding the optical density at each wave-length of the free 4-amino-5-carboxamidoimidazole or the IRP and that of adenine at the corresponding pH are almost identical with the curves of the authentic IRPA. However, the molar extinction at 260 mμ was somewhat smaller than the sum of the molar extinctions of the imidazole and of adenine. The absorption curve of IRP differed slightly from that of the free imidazole at pH 2.6. The latter showed a small but definite maximum at 240 mμ which did not appear in the curve for IRP. Instead there was a small shoulder at about 250 mμ. The maximum was in both cases at 267 mμ.

Behavior of IRPA, IRP, and of Synthetic 3-Benzyl-4-amino-5-carboxamidoimidazole in Bratton-Marshall Test—The color given by IRPA or IRP in the Bratton-Marshall test was purple, whereas that given by free 4-amino-5-carboxamidoimidazole was red. The differences in absorption spectra can be seen in Fig. 6. The peaks for the dyes from IRPA and IRP are displaced towards greater wave-lengths, with respect to the absorption spectrum of the azo dye of the free 4-amino-5-carboxamidoimidazole. The ratio of optical densities at 540 and 600 mμ was a useful diagnostic tool in distinguishing between the free imidazole and the nucleotides. This ratio for the imidazole was 3.50, for IRP it was 1.82, and for IRPA 1.72.

[7] A semipurified preparation, kindly supplied by Dr. M. Kunitz.

These differences in absorption arose from the possession of a group in position 3 of the imidazole ring. A model compound was very kindly supplied by Dr. E. Shaw of this laboratory, which was synthetic 3-benzyl-4-amino-5-carboxamidoimidazole. This model compound also gave a purplish color with the Bratton-Marshall test. The absorption spectrum of this azo derivative was almost identical with those given by the azo derivatives of IRPA and IRP. A further study showed that the color yield

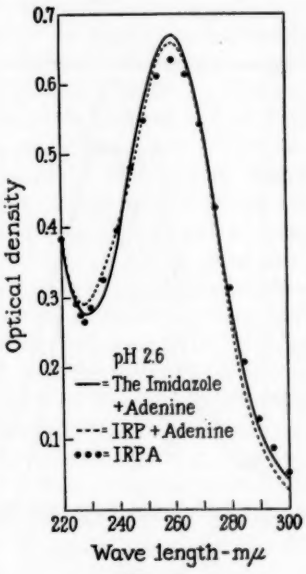

FIG. 3

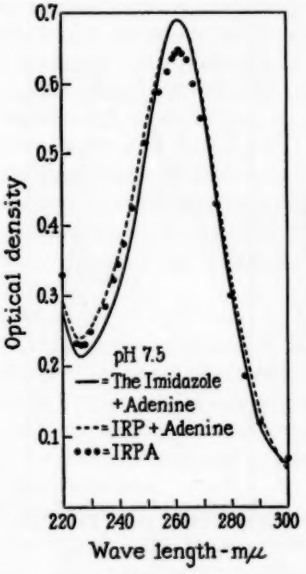

FIG. 4

FIG. 3. Ultraviolet absorption spectrum of IRPA and of its components at pH 2.6 and at 2.74×10^{-5} M.

FIG. 4. Ultraviolet absorption spectrum of IRPA and of its components at pH 7.5 and at 2.74×10^{-5} M.

from the free imidazole differed from that obtained from the imidazoles with substituents in position 3. A quantitative measure of this difference was made by carrying out the color reactions of IRPA and of IRP in such a way that the naphthylethylenediamine reagent was the limiting reactant rather than the imidazole compound. By use of a constant excess of IRPA or IRP, large in comparison to the varying amounts of the naphthylethylenediamine (at least 10-fold), the color yield of IRPA or IRP could be determined in relation to free 4-amino-5-carboxamidoimidazole irre-

spective of the absolute purity of the nucleotides.[8] These experiments showed that the molar extinctions at 540 mμ of the azo dyes of IRPA and IRP were 83 per cent of the molar extinction of the free 4-amino-5-carboxamidoimidazole. The synthetic model compound 3-benzyl-4-amino-5-carboxamidoimidazole showed this same kind of behavior. Consequently this number was applied as a correction to obtain the values for imidazole content presented in Table II.

Evidence That IRPA Was Not An "Onium" Compound—Aside from the improbability from the stand-point of organic chemistry that IRPA could be an "onium" compound, there was direct experimental evidence to show that it was not, namely the behavior of IRPA (and of IRP derived therefrom) in electrophoresis. As explained above for IRP, the IRPA would have migrated much more slowly if it had contained the highly ionized "onium" linkage.

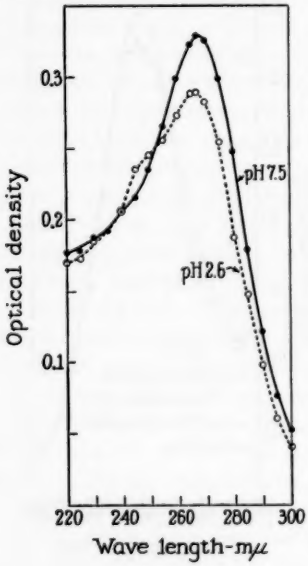

FIG. 5

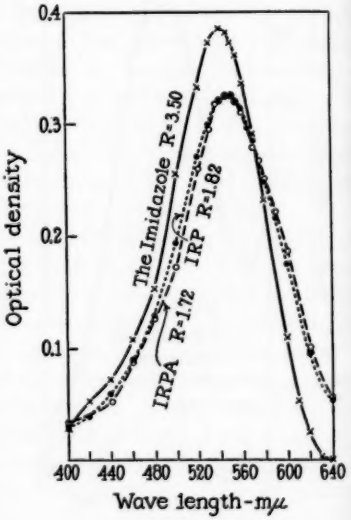

FIG. 6

FIG. 5. Ultraviolet absorption spectra of IRP (2.74×10^{-5} M) at pH 2.6 and 7.5.

FIG. 6. Spectra of the azo derivative of the imidazole, IRP, and IRPA, each at a concentration of 8×10^{-6} M.

[8] Either IRP or IRPA developed a purplish color without any addition of coupling amine. This complication was overcome by the use of appropriate controls. Diazotized free 4-amino-5-carboxamidoimidazole did not give color prior to the addition of coupling amine.

Position of Ribosidic Linkage in IRPA—There was no direct proof that the ribose was attached to position 3 of the imidazole rather than to position 1. It was tentatively placed at position 3 from biological considerations, since purine nucleosides, for which IRPA may be a precursor, have the ribose attached at the position corresponding to 3 in the imidazole. The ribose could not have been on the $—NH_2$ group because such a compound would not give a Bratton-Marshall test.

Hydrolysis of IRPA with HCl—The course of liberation of the free imidazole from IRPA during acid hydrolysis was followed by measuring the change in the ratio of optical density at 540 and 600 mμ of the Bratton-Marshall color. As described above, the imidazole had a ratio of 3.50 and the IRPA 1.72. With 1 N HCl at 100° for 15 minutes, 25 per cent of the imidazole was liberated. With 2 N HCl for the same time and temperature 40 per cent was set free. Complete hydrolysis occurred in 2 N HCl for 1 hour at 100°. Although the imidazole by itself was not destroyed by 2 N HCl for 1 hour at 100° (95 per cent recovery), about 20 per cent of that liberated from IRPA was lost under these conditions. The hydrolysis of IRP and the identification of two of the products as the imidazole and ribose-5-phosphate were described earlier in this paper.

Failure of DPNase to Attack IRPA—0.41 μmole of IRPA and 13 units of purified DPNase at pH 7.45 (Tris) in 0.15 ml. were incubated 24 hours at 38°, and the reaction mixture was separated by paper electrophoresis. 0.44 μmole of the dinucleotide exhibiting the proper rate of migration for IRPA and no free imidazole were recovered. An appropriate control with no enzyme added gave identical results. Paper chromatographic separations confirmed the results obtained by electrophoresis.

Inhibition of IRPA Synthesis by Nicotinamide—The question might arise as to whether DPNase was the enzyme responsible for the synthesis of IRPA, for, although the DPNase was much purified, it was probably not a single protein. Inhibition of the synthesis by nicotinamide would be evidence that the enzyme concerned was really DPNase. When the reaction for synthesis was carried out as described above, except that the solution was 0.02 M with respect to nicotinamide, no IRPA could be detected in the proper eluates of the paper sheets after the mixture had been subjected to electrophoresis.

Was Enzyme Necessary for Synthesis?—Many controls were run in which the enzyme was omitted from the reaction mixture. No IRPA could be detected under these conditions.

Similarly, controls were run with enzyme, but without 4-amino-5-carboxamidoimidazole, to test the idea that some impurity in the enzyme reacted with DPN to give rise to IRPA. No diazotizable amine, and hence no IRPA, was detected in these controls. Furthermore, no IRPA was

formed from enzyme plus the imidazole plus the nicotinamide-free hydrolytic product of DPN (RPA).

DISCUSSION

Because one of the substrates of this reaction (*viz.* 4-amino-5-carboxamidoimidazole) is a recognized intermediate in the *de novo* synthesis of the purines, it is possible that the reaction here described is of importance in purine biosynthesis. Thus, it may be that the new dinucleotide is a physiological substrate for enzymes which insert a formyl group (donated from a folic acid derivative) to yield a dinucleotide of inosinic and adenylic acids. Cleavage of this compound by nucleotide pyrophosphatase would give inosinic acid directly. Another possibility is that IRPA is formed and then cleaved by nucleotide pyrophosphatase to IRP plus AMP. The IRP has already been demonstrated by Greenberg (24) and by Buchanan and Wilson (25) to yield inosinic acid in crude enzyme preparations.

However, if IRPA did give rise to the inosinic-adenylic dinucleotide, this latter compound would possess the energy-rich pyrophosphate bond which might be used to esterify an existing polynucleotide at 3′ positions, thereby lengthening the nucleotide chain by 1 residue.

Current beliefs, derived from the work of Greenberg *et al.* (16, 26) and of Hartman *et al.* (27), are not entirely in accord with such an idea because they picture the ribose as being attached to the glycinamide portion of the future imidazole nucleotide before the imidazole ring is formed. This would seem to exclude the formation and participation of free 4-amino-5-carboxamidoimidazole in purine biosynthesis. Nevertheless, administration of labeled 4-amino-5-carboxamidoimidazole to animals does lead to formation of nucleic acids containing labeled purines (28). It may well be that there are several routes of purine formation, one of which involves ribosidation of a glycinamide derivative and another which goes through the DPN reaction pathway starting with the free imidazole. Then, too, the systems which have been used to study purine biosynthesis are frequently so unpurified that they might well leave room for the presence of both DPN and DPNase.

From a practical stand-point the reactions described in this paper provide a relatively easy method for the preparation of weighable quantities of IRP. This imidazole mononucleotide has previously been obtained only with great difficulty.

SUMMARY

Soluble, purified beef spleen DPNase was found to catalyze a reaction between cozymase (DPN) and 4-amino-5-carboxamidoimidazole which led

to the formation of a new dinucleotide. This new dinucleotide, IRPA, was isolated in apparently pure form, and its structure was established by suitable analyses and degradations. It was derived from DPN by replacement of the nicotinamide moiety by the 4-amino-5-carboxamidoimidazole. The quaternary ammonium linkage present in DPN was absent in the new dinucleotide. The reaction was shown to differ from the base-exchanges previously recognized. In these exchanges another pyridine derivative replaces the nicotinamide in DPN without loss of the "onium" linkage. The new reaction was sought as an example of a novel class of biosynthetic mechanism, *viz.* those which seem to be energized by reduction of an "onium" linkage.

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METABOLISM OF GLYCOLALDEHYDE IN THE RAT*

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Though glycolaldehyde has long been considered as a possible metabolic intermediate (1), its importance in this respect has been enhanced in recent years with the discovery that an active enzyme complex of this substance is involved in the interconversion of sugars by way of the transketolase reaction (2–4). It has also been suggested as an intermediary in the conversion of ethanolamine and pentose to acetate (5) and of ethanolamine to glycine (6). In the present report a method is described for the preparation of glycolaldehyde-2-C^{14} ($C^{14}H_2OHCHO$), together with results of experiments bearing on its significance in the intermediary metabolism of the rat.

The following metabolic transformations were studied: to glycine by using the hippuric acid technique (7), to CO_2 by collection of respiratory CO_2 in alkali, to oxalic and formic acids by "trapping" techniques described previously (8), to acetate by trapping either as the α-amino-γ-phenylbutyric acid derivative *in vivo* (9) or as acetoacetate in liver slices *in vitro* (10), and to glycogen by a standard procedure (11).

Methods and Results

Preparation of Glycolaldehyde-2-C^{14}—The reaction is carried out in a 3-necked flask equipped with a dropping funnel, a mechanical stirrer, and a condenser leading to a bead tower filled with sodium hydroxide for absorption of CO_2. The top of the condenser is connected to a vacuum line and gentle suction is applied throughout the reaction period to draw off CO_2.

Serine-3-C^{14}, in an amount of 10 mmoles, is dissolved in 50 ml. of water, the solution is placed in the 100 ml. 3-necked flask, and, while stirring me-

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† The work related to glycogen isolation and degradation will constitute part of a thesis to be submitted by Herman Levin to the Graduate Council of Temple University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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chanically, 0.75 ml. of 85 per cent phosphoric acid is added, followed by 3.60 gm. (20 mmoles) of ninhydrin. The flask is then heated by means of a water bath at 55–60° until the supernatant solution is colorless (about 90 minutes). The precipitate is filtered and the filtrate is shaken for a few minutes with about 2 gm. of Darco absorbent charcoal. The treatment with charcoal is repeated once and the supernatant solution is shaken with successive portions of Amberlite MB-3 ion exchange monobed resin until the blue color of the resin persists. This treatment removes excess ninhydrin, ammonium phosphate, and phosphoric acid. The only carbon-containing materials present at this stage are glycolaldehyde and about 5 per cent glyoxal. The yield is 80 to 85 per cent. Although it was not found possible to isolate this product in pure form, the identity of the material in solution was established by means of the following observations.

The solution is free of nitrogen determinable by the Kjeldahl reaction, does not give any color with ninhydrin, and yields no ninhydrin-sensitive spots when chromatographed on paper. When an aliquot of this solution was oxidized with persulfate (12), the resultant CO_2 had a specific activity exactly 1.5 times that of the original serine. The content of bisulfite-binding material checked closely with the carbon content as determined by persulfate oxidation. On oxidation with periodate according to the method of Reeves (13), the calculated yields of formaldehyde and formic acid were obtained. The specific activity of the formaldehyde was twice that of the whole solution, whereas the formic acid had only 3 to 7 per cent of the activity of the formaldehyde. These findings are consistent with the probability that the solution contains essentially only glycolaldehyde-2-C^{14} together with a small quantity of glyoxal.

On treatment with dimedon according to the procedure of Goepfert and Nord (14), the solution yields a crystalline product, m.p. 218°, as compared with a reported value for the addition product of 224–226° (14). On treatment with excess 2,4-dinitrophenylhydrazine in 2 N HCl it yields the osazone, m.p. 322°; reported figure, 329° (15).

Glycine Formation—In all of the experiments to be reported, young, adult, male rats of our stock colony Carworth Farms strain, weighing about 200 gm., were used. These were routinely fed on Allied Mills rat checkers and were fasted for 24 hours prior to their use in these experiments in order to avoid complications due to exogenous metabolism. The experiments were conducted exactly as described previously for other 2-carbon acids (7). A solution of the labeled glycolaldehyde, in an amount between 0.2 and 0.4 mmole, was injected intraperitoneally, along with 1 mmole of sodium benzoate. The rat was then placed in a metabolism chamber where respiratory CO_2 was collected during the ensuing 6 hours. The urine, collected for 24 hours, was treated for the isolation of hippuric and oxalic acids.

The results of four such experiments are listed in Table I. From 5 to 15 per cent of the administered aldehyde was converted to glycine of hippuric acid, from 9 to 12 per cent was oxidized to CO_2, and about 0.5 per cent was converted to oxalic acid. Reference to a previous publication (7) reveals that this metabolic behavior is strikingly similar to that of glycolic and glyoxylic acids and that of glycine itself. Weissbach and Sprinson (6) have shown that ethanolamine-1-C^{14} readily yields glycine-1-C^{14} in the rat and suggested that this transformation proceeds via glycolaldehyde and glycolic and glyoxylic acids. Pilgeram *et al.* (16) and Abbott and Klingman (17) also found a limited conversion of ethanolamine to glycine in the rat, and Kun *et al.* (18) have also shown recently that glycolaldehyde is oxidized

TABLE I

Metabolic Transformation of Glycolaldehyde-2-C^{14} in Intact Rat

The experimental details are given in the text.

Experiment No.	Glycolaldehyde, amount injected	Co metabolite added	Glycolaldehyde activity, per cent recovered in			
			Hippuric acid	Oxalic acid	Carbon dioxide	Formic acid
	mmole					
1	0.31	Sodium benzoate	5.7		9.4	
2	0.31	" "	5.5	0.5	12.4	
3	0.37	" "	12.1	0.5	8.9	
4	0.37	" "	15.5	0.4	10.2	
5	0.23	" formate		2.0	13.9	1.30
6	0.23	" "			12.0	1.29

to glycolic and glyoxylic acids in rat liver. The present results, in conjunction with these preceding studies, indicate that there is a rapid conversion of glycolaldehyde to glycine in the rat. That this is a direct process is further indicated by our observation that on decarboxylation of one of these glycines with ninhydrin essentially all (96 per cent) of the activity of glycolaldehyde-2-C^{14} was found in carbon 2 of glycine.

Conversion to Formic Acid—It was already found in earlier experiments in which the "trapping" technique was employed (8) that the α-carbons of glycolic and glyoxylic acids, as well as of glycine, were slowly converted to formic acid in the rat. Two similar experiments were conducted with glycolaldehyde-2-C^{14}. The results, as shown in Experiments 5 and 6 of Table I, again indicate the similarity in the metabolic routes of glycolaldehyde and these other 2-carbon acids; under the conditions used, from 0.5 to 2.5 per cent of these acids was converted to formate, whereas about 1.3 per cent of glycolaldehyde was thus converted.

Oxidation to CO_2—In all of the experiments cited, approximately 10 per cent of the administered labeled glycolaldehyde carbon was oxidized to CO_2 in the 6 hour CO_2 collection period (Table I). The excretion curves followed closely those obtained in similar previous experiments with glycine, glycolate, and glyoxylate (8), further emphasizing the close metabolic relationships of these substances. From these and the previous results (8, 10) a probable major pathway for the combustion of glycolaldehyde is the following: glycolaldehyde → glycolic acid → glyoxylic acid → CO_2 + formic acid → CO_2.

Unknown Products of Glycolaldehyde Metabolism—The above mentioned products account for only about 25 per cent of the total administered glycolaldehyde. In view of the rapid conversion to glycine, it may be assumed that the glycine excreted as hippuric acid represents a minimal value which may be far below the actual conversion to this substance; much glycine must also have been incorporated into protein or undergone metabolism to other products. Oxidation of the total urinary carbon by means of persulfate (12) disclosed that about 13 per cent of the dose was present in the urine in unknown form. Experiments with added carriers indicated that it was not present in appreciable amounts as urea, carbonate, creatine or creatinine, glucose, or glycolaldehyde. No free glycine or other amino acids were detectable in greater than normal amounts. The identity of this urinary glycolaldehyde metabolite is being investigated further.

Acetate Formation—To ascertain the extent of acetate formation from glycolaldehyde in the intact rat, the incorporation of glycolaldehyde carbon into the acetyl derivative of α-amino-γ-phenylbutyric acid[1] was studied according to the procedure of Bloch and Rittenberg (9). A male rat weighing 165 gm. was given 0.28 mmole of glycolaldehyde-2-C^{14} intraperitoneally and 200 mg. of sodium DL-α-amino-γ-phenylbutyrate by stomach tube. The aldehyde had a specific activity of 20,500 c.p.m. In 6 hours 16.5 per cent of the activity was excreted in the respiratory CO_2. From the urine excreted in 6 hours, 50 mg. of the acetyl derivative were obtained according to the procedure of Bloch and Rittenberg (9). To this were added 151 mg. of non-isotopic acetyl derivative, giving a mixture with an activity of 1500 c.p.m. On recrystallization the activity dropped to 29 c.p.m. and on a second crystallization it dropped to 3 c.p.m. An additional 116 mg. of acetyl derivative were isolated from the 6 to 24 hour urine collection, having an activity of 28 c.p.m.; on recrystallization the activity dropped to zero.

In a second experiment, conducted similarly, 150 mg. of acetyl derivative were isolated from the 24 hour urine collection. 1 mmole of non-isotopic sodium acetate was added as a carrier, and the acetyl group was hydrolyzed by prolonged boiling with 20 per cent NaOH. The solution

[1] We are grateful to Dr. David Sprinson for kindly supplying this material.

was acidified, and the acetic acid was distilled, yielding 1.47 mmoles. This was neutralized with sodium hydroxide and evaporated to dryness. It had no measurable radioactivity.

To test further the occurrence of this transformation, and to learn something of the metabolic behavior of glycolaldehyde *in vitro*, an experiment was conducted with rat liver slices, in the manner employed previously to ascertain the extent of conversion of glycine to acetyl groups (10). The transformation of glycolaldehyde-2-C^{14} and acetate-2-C^{14} to CO_2, and to acetoacetate, fatty acids, and "unsaponifiable lipide," was estimated (19). The results of this experiment are shown in Table II. As expected from

TABLE II

Metabolic Activity of Glycolaldehyde-2-C^{14} in Rat Liver Slices

The experiments and calculations were conducted exactly as described previously (19) with approximately 5 gm. of tissue slices and an incubation time of 4 hours at 37°. The oxygen uptake values for acetate-2-C^{14} and glycolaldehyde-2-C^{14} are 920 and 740 μmoles, respectively. The values are given in microatoms of substrate carbon converted.

Metabolic conversion	Substrate	
	Acetate-2-C^{14}	Glycolaldehyde-2-C^{14}
Carbon dioxide	9.75	2.56
Acetoacetic acid	11.2	0.02
Fatty acids	1.93	0.04
Unsaponifiable lipides*	725	12

* Since no attempt was made to isolate pure cholesterol, the values given are of the unsaponifiable fraction as such in counts per minute per standard dish, as determined by our usual counting procedure (19).

previous work (19), acetate underwent all four transformations readily. However, glycolaldehyde, though oxidized to CO_2, was only negligibly converted to acetoacetate, fatty acids, or cholesterol. It has already been established that an acetyl precursor such as lactic acid readily yields acetyl groups for acetoacetate formation in liver slices, whereas glycine does not (10). It can therefore be concluded that glycolaldehyde is not readily converted to acetyl groups in the rat liver slice.

Conversion to Glycogen—Two male rats, fasted 24 hours, were each given an intraperitoneal injection of glycolaldehyde in 1 ml. of saline solution, and at the same time given 5 mmoles of non-isotopic glucose in 3 ml. of water by stomach tube. The animals were sacrificed 4 hours later, the livers were excised, and the glycogen was isolated therefrom by the procedure of Good, Kramer, and Somogyi, as modified by Boxer and Stetten (11). For comparison, similar experiments were conducted with glucose-C^{14} and with

labeled lactic and pyruvic acids, serine, and glycine. The amounts administered, the type of labeling, and the results obtained are listed in Table III. Glycolaldehyde was converted to glycogen in the rat; however, the rate, as judged by the incorporation of labeled carbon, was relatively low when compared with that of carbon from pyruvate or from serine or glycine. In view of the rapid conversion of glycolaldehyde to glycine, already observed, the data indicated that probably no direct incorporation of glycolaldehyde occurred, but that it passed through the stages of glycine, serine, and triose in its conversion to glycogen.

This interpretation is borne out by the results of the degradation of these glycogens, whereby the distribution of labeled carbon in each of the carbons of the glucose molecule was ascertained.

Degradation of Glycogen—An amount of glycogen corresponding to 1 mmole of glucose is hydrolyzed by boiling in 2 N H_2SO_4 for 2 hours, and the solution is cooled and neutralized. It is then added to 150 ml. of 0.05 M phosphate buffer, pH 6.5, contained in a 300 ml. flask carrying a lead in tube and connected with a bead tower for CO_2 absorption in 0.5 M NaOH.

The bacteria, a strain of *Leuconostoc mesenteroides* kindly provided by Dr. Martin Gibbs,[2] were cultured as described by Gunsalus and Gibbs (20). The cells are grown in 10 ml. batches and used to inoculate 125 ml. of broth, which is incubated at room temperature (about 30°). When gas evolution becomes vigorous (10 to 14 hours), the bacteria are centrifuged, washed once with distilled water, and recentrifuged. Washed cells from 250 ml. of broth are added to the glucose solution and the contents are incubated at room temperature for 5 hours. During this time nitrogen, washed with alkali and pyrogallol, is passed through the solution and into the bead tower.

The fermentation occurs quantitatively, and without appreciable dilution by endogenous bacterial carbon, as follows:

$$\overset{6}{CH_3}\overset{5}{OHCHOH}\overset{4}{CHOH}\overset{3}{CHOH}\overset{2}{CHOH}\overset{1}{CHO} \rightarrow$$

$$\overset{6}{CH_3}\overset{5}{CHOH}\overset{4}{COOH} + \overset{2}{CH_3}\overset{3}{CH_2}\overset{1}{OH} + CO_2$$

Isolation of the CO_2 and further degradation of the ethanol and lactic acid afford a simple and reliable method for separate assays of each glucose carbon. These were conducted in the following way.

The bacteria are centrifuged out of the incubation mixture, which is then made basic with NaOH. The ethanol is separated from the lactate by distillation into an ice-cooled flask containing 1 gm. of potassium dichromate. The distillate is made 4 N with H_2SO_4 and then heated for 2 hours

[2] Our thanks are due to Dr. Martin Gibbs for supplying detailed directions for the growth of the bacteria and their use in glucose fermentation.

at 90° in an oven (21). The resulting acetic acid is steam-distilled twice, converted to the sodium salt, and decarboxylated by the Phares modification of the Schmidt reaction (22), the CO_2 being trapped as usual in a bead tower containing CO_2-free NaOH. The solution containing the residual methylamine is made basic with NaOH and the liberated methylamine is drawn by suction into a bead tower containing an excess of 0.5 N H_2SO_4. It is then oxidized with persulfate (12) and the CO_2 is collected and assayed for radioactivity.

The basic, residual solution containing sodium lactate is evaporated to a small volume, acidified, and extracted continuously for 24 hours with ether. The ether is evaporated and the lactic acid is oxidized by potassium permanganate and degraded according to the procedure of Friedmann and Kendall (23) as modified by Lorber.[3]

The CO_2 thus obtained from the lactic acid is derived from glucose carbon 4, and the next 2 carbons, corresponding to the 5 and 6 carbons of glucose, are trapped as acetaldehyde in a bead tower containing sodium bisulfite. The aldehyde-bisulfite complex is broken up with $K_2HPO_4 \cdot 7H_2O$ and the aldehyde is collected in water in a cooled flask containing 1 gm. of potassium dichromate. This solution is made 4 N with H_2SO_4 and heated for 2 hours at 90° in an oven. The resulting acetic acid is degraded as described above. These procedures were tested with labeled glucose, lactates, and acetates and found to yield reliable results. A detailed description of this degradation procedure, as well as tests of its reliability, has been published recently by Bernstein *et al.* (24).

Distribution of Precursor Carbons in Glycogen[4]—The studies of Lorber *et al.* (25), Topper and Hastings (26), and Landau *et al.* (27) are in good agreement in indicating that, in the formation of glycogen from pyruvic acid, the immediate precursors of the hexose chain are two 3-carbon compounds completely or very nearly in equilibrium. These couple in such a manner that pyruvate carboxyl carbons appear only in glucose carbons 3 and 4. Also, carbons 2 and 3 of pyruvate become nearly completely randomized among carbons 1, 2, 5, and 6 of glucose, and both of these pyruvate carbons also migrate appreciably to positions 3 and 4 of glucose. For example, Lorber *et al.* (25) found that the administration of lactate-3-C^{14} to rats *in vivo* gave a C^{14} distribution of the following type in glucose from liver glycogen:

$$\overset{24}{C}—\overset{20}{C}—\overset{6}{C}—\overset{6}{C}—\overset{20}{C}—\overset{24}{C}$$

Landau *et al.* (27) found that glycogen

[3] Lorber, V., private communication.

[4] In this section the distribution of C^{14} in glucose is portrayed in percentage of the total contained in each glucose carbon; where differentiation has been made the numbering of the glucose carbons proceeds from left to right, as in Table IV. The term "symmetry" in reference to glycogen in this paper means a symmetry of C^{14} distribution in glucose carbons, such that carbons 3, 2, and 1, respectively, have the same activities as carbons 4, 5, and 6, respectively.

synthesized by rat liver slices in the presence of pyruvate-2-C^{14} had the following C^{14} distribution in its glucose carbons: C—C—C—C—C—C. These distributions are similar on the whole to those obtained in the present study, by using a degradation procedure which allows an independent assay of each glucose carbon.[5] Administration of pyruvate-3-C^{14} led to the following essentially symmetrical isotope distribution as shown in Table III: C—C—C—C—C—C. From lactate-2-C^{14}, an appreciable quantity

TABLE III

Conversion of Glycolaldehyde-2-C^{14} and Other Labeled Substances to Glycogen in Intact Rat

Labeled substances were administered intraperitoneally while at the same time 10 mmoles of unlabeled glucose were given by stomach tube. 4 hours later, the animals were sacrificed and liver glycogen was isolated and assayed.

Substance injected	Amount injected	Liver glycogen		
		Amount isolated calculated as glucose	Relative specific activity	Administered dose converted
	mmoles	*mmoles*	*per cent*	*per cent*
Glycolaldehyde-2-C^{14}	0.50	2.10	0.18	2.2
"	0.57	2.32	0.17	2.3
Glucose-U-C^{14}*	10.10	2.08	42.8	15.0
"	9.80	2.40	48.8	12.0
Pyruvic acid-3-C^{14}	0.52	1.98	0.59	4.5
Serine-3-C^{14}	0.49	2.02	0.57	4.7
Glycine-2-C^{14}	0.66	1.74	0.38	3.9

* Uniformly labeled glucose.

of isotope appeared in carbons 1, 3, 4, and 6 in addition to the expected preponderance of activity in carbons 2 and 5, *e.g.* C—C—C—C—C—C.

The formation of a glycogen whose glucoses are symmetrically labeled around carbons 3 and 4 may be regarded as evidence of a 3-carbon stage in their synthesis. In previous studies on the rat by Sakami (30) and on the

[5] In contrast with the results of this study and those cited, Schambye *et al.* (28), using the same degradation procedure, found that glycogen arising from glycerol-1-C^{14} administered to rats had 1.4 to 2.4 times as much activity in carbon 3 as in carbon 4. Results consistent with an asymmetric distribution of glycerol carbons in liver glycogen were also reported by Landau *et al.* (27); however, a symmetrical distribution of glycerol carbon in rat liver glycogen was observed in similar experiments by Doerschuk (29).

chick by Bernstein (31), it was found that glycine yielded liver glycogen with a symmetrical distribution around carbons 3 and 4, and this was attributed to the intermediary intervention of 3-carbon compounds such as serine and pyruvate in this process. On this basis, symmetrical labeling in glycogens obtained in these experiments from glycine-1- and 2-C^{14} indicates that most or all of the glycine is transformed via 3-carbon intermediates. That serine represents one of these steps is further indicated by the symmetrically labeled glycogen derived from serine-3-C^{14}, *viz.* C—C—C—C—C—C.[6]

With glycine-2-C^{14}, the glycogen-C^{14} distribution was C—C—C—C—C—C; with glycolaldehyde, it was C—C—C—C—C—C. These essentially identical distribution patterns, in the light of this discussion, strongly indicate a common path for glycogen formation from glycolaldehyde and glycine via serine and triose. If glycolaldehyde as such were readily activated for participation in transketolase reactions, a preponderance of its activity should have appeared in carbons 1 and 2 of hexose (2, 3). Thus it appears either that the activation of glycolaldehyde to form an active enzyme complex may be limiting, or that the transketolase reactions may be relatively slow compared with the glycine-serine-triose pathway.

[6] It has been pointed out previously by Lorber *et al.* (25), by Topper and Hastings (26), and by Landau *et al.* (27) that most of the pyruvate undergoing conversion to glycogen does so indirectly, after first undergoing reactions (carboxylation, citric acid cycle, conversion to acetate, etc.) leading to randomization of carbons 2 or 3 among all 3 carbons. From previous considerations (25–27), in glucose formation from pyruvate-3-C^{14}, the difference in percentage of C^{14} between glucose carbons (1 + 6) and (2 + 5) is a measure of the direct phosphorylation of pyruvate and reversal of glycolysis. This pathway is relatively small with pyruvate-3-C^{14}; the difference between (24 + 22) and (18 + 22) = 6 per cent. With lactate-2-C^{14} the difference between ($C_2 + C_3$) and ($C_1 + C_3$) was somewhat higher, *viz.* 49 − 36 = 13 per cent. With serine, the picture was quite different; randomization was far less complete, and the difference in this instance between ($C_1 + C_3$) and ($C_2 + C_3$) was 66 − 24 = 42 per cent. This *direct* conversion of almost half of the serine molecules to glucose suggests that pyruvate as such may be largely by-passed in the conversion of serine to glycogen. It is possible that serine undergoes transamination to hydroxypyruvic acid and thus enters the glycolytic process at a higher stage than pyruvate, possibly via glyceric acid (32, 33). The experiment cited is ambiguous, however, since DL-serine-3-C^{14} was used. The distribution of carbon 3 of D- and L-serine in glycogen is now under further investigation; if the low degree of randomization is confirmed for L-serine, this will indicate that serine carbon enters the pathways of sugar metabolism above the pyruvate stage and will enable the reconciliation of the glycogenic activity of serine with its sluggishness in forming acetyl groups.

The possibility was considered that unsymmetrically labeled glucose may have been formed in these experiments, but underwent a subsequent randomization. However, this was rendered unlikely by the observation, as shown in Table IV, that glucose-1-C^{14} under similar conditions yielded glycogen with 79 per cent of its activity in carbon 1. Thus any asymmetry of labeling, had it occurred, should have been detected. This finding is in accord with the pattern of glycogen labeling observed by Hers (34) after administration of glucose-1-C^{14}, and by Bernstein *et al.* (24) after administration of glucose-2- or 6-C^{14}.

TABLE IV

Distribution of C^{14} among Glucose Carbons of Liver Glycogen Isolated after Administration of C^{14}-Labeled Precursors

The values for each carbon are given in per cent of total radioactivity in glycogen.

Substance injected	Glucose carbon No.					
	1	2	3	4	5	6
Glycolaldehyde-2-C^{14}	21	26	6	7	22	19
Glycine-2-C^{14}	20	26	5	6	24	20
Serine-3-C^{14}	34	12	5	5	12	32
Pyruvic acid-3-C^{14}	24	18	7	8	22	22
Lactic acid-2-C^{14}	17	24	7	8	25	19
Glucose-U-C^{14}	17	22	19	16	15	21
Glucose-1-C^{14}	79	4	3	3	4	8

DISCUSSION

Glycolaldehyde, having been isolated by Neuberg and Rosenthal as a product of fermentation by yeast (35), was suggested very early as a possible metabolic intermediate (1, 35). More recently it was found to be a product of metabolism of ethylene glycol in fusaria (14) and in *Acetobacter acetigenum* (36) and was also obtained in culture media of the latter organism growing on D-xylose or L-arabinose (10). As far as we are aware, these are the only recorded instances of its isolation from biological sources. However, it is becoming increasingly apparent that a complex of diphosphothiamine and glycolaldehyde is transiently formed in a variety of transketolase reactions (4). If this complex has an independent existence, it might serve as an intermediate in the transformation of sugars to glycine or to acetyl groups and might conceivably also provide a path whereby glycine, acetate, or related substances may be interconverted or be transformed to sugars, as shown in the accompanying diagram.

$$\begin{matrix}\text{Hexose} \leftrightarrows \\ \text{Pentose} \rightleftarrows\end{matrix} \text{glycolaldehyde} \begin{matrix}\leftrightarrows \text{ acetyl groups} \\ \rightleftarrows \text{ glycine}\end{matrix}$$

Gest and Lampen (37) observed the conversion of D-xylose-1-C^{14} to acetate-2-C^{14} in *Lactobacillus pentosus*, and a similar transformation of L-arabinose-1-C^{14} was reported by Rappaport *et al.* (38). Sprinson and Weliky (5) reported that D-ribose-1-C^{14} or D-xylose-1-C^{14} gave high yields of acetate-2-C^{14} in the intact rat and suggested glycolaldehyde as an intermediate. However, the present data appear to exclude glycolaldehyde as such as an efficient precursor of acetyl groups in the rat.

Weissbach and Horecker (39) observed that extracts of spinach leaves converted ribose to glycine. However, Greenberg and Sassenrath (40) could not detect the formation of C^{14}-labeled glycine in the rat from arabinose-1-C^{14} or ribose-1-C^{14}. In our own laboratory, ribose-1-C^{14} yielded a small amount of labeled glycine for hippuric acid formation in the intact rat, but the amount was of the same order as that given by labeled hexoses.[7]

Regarding the reverse transformations, von Euler (41), Forrest *et al.* (42), and McGeown and Malpress (43) observed pentose formation from glycolaldehyde and trioses in various tissue preparations. Bernstein also has presented isotopic tracer evidence to indicate that probably not all of the glycine and acetate carbons converted to ribose in the chick follow a pathway involving hexose and has suggested a direct $C_2 + C_3$ condensation as an alternative (31, 44). However, a similar direct involvement of a C_2 unit in hexose formation was contraindicated, as it is in the present experiments, by the symmetrical distribution of both glycine and acetate carbons in the liver glycogen.

Anker (45) and Högstrom (46) observed a low incorporation of labeled acetate into hippuric acid glycine in rats fed sodium benzoate. However, both investigators discounted a direct transformation, finding that their data were in accord with an indirect pathway for this transformation; *e.g.*, acetate → oxalacetate → pyruvate → serine → glycine, rather than a direct path such as acetate → glycolaldehyde → glycine.

Attempts by early investigators to determine the glycogenic capacity of glycolaldehyde by balance experiments in intact animals were inconclusive and contradictory (1, 47), owing principally to the toxicity of glycolaldehyde, which prevents its administration in sufficiently large amounts to obtain decisive results. Though the present data clearly indicate that glycolaldehyde is "glycogenic," when they are considered in the light of these previous studies, there appears to be little evidence for the participation of glycolaldehyde as such in the metabolism of either hexoses or acetate. The only gateway now experimentally demonstrable for the entry of glycolaldehyde carbon into general metabolism is glycine, and its appearance in glycogen stems presumably from a metabolic sequence of the type glycine → serine → pyruvate (or hydroxypyruvate) → glucose. If more direct path-

[7] Unpublished observations.

ways are utilized for sugar or acetate formation from glycolaldehyde, these must occur at a relatively low rate.

SUMMARY

Glycolaldehyde-2-C^{14} was synthesized by decarboxylation of DL-serine-3-C^{14}, and its metabolism was studied in the rat. It was found to be oxidized readily to CO_2 and to glycine and to be converted slowly to formic and oxalic acids and glycogen, but was not converted appreciably to acetate. A symmetrical distribution of glycolaldehyde carbon 2 in glycogen, the same as was observed for glycine carbon 2, clearly indicated that a 3-carbon intermediary is involved in the conversion of both substances to glycogen, and that glycine is probably an intermediate in glycogenesis from glycolaldehyde. The data do not support the likelihood of an appreciable participation of administered glycolaldehyde as such in reactions involving transketolase.

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METABOLIC PATHWAYS OF HOMOSERINE IN THE MAMMAL*

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The importance of homoserine in the metabolism of amino acids in a variety of living forms is now well established. This amino acid was found to be the common precursor of methionine and threonine in *Neurospora crassa* (1). Enzymes involved in the conversion of aspartic acid to threonine via homoserine have been studied in *Escherichia coli* (2–5) and in bakers' yeast (6–8). The presence of homoserine (9) and its derivatives (10, 11) has been demonstrated in various plants. In intact rats at least a part of the reported *de novo* synthesis of methionine seems to utilize the carbon skeleton of homoserine lactone (12). The metabolic formation of homoserine in mammals from methionine has been suggested from the study of the cleavage of cystathionine (13), and evidence for such conversion has been reported from this laboratory (14). In continuation of the studies on the metabolic fate of the main carbon chain of methionine, the investigation reported in this paper was undertaken in order to clarify the course of homoserine metabolism.

Using DL-homoserine-2-C^{14}, we have been able to isolate and identify α-ketobutyric, α-hydroxybutyric, α-amino-*n*-butyric, and propionic acids by chromatographic means (*cf.* Figs. 1 and 2, and Table I).

That homoserine is converted into α-ketobutyric acid in a rat liver system has been reported previously by Carroll, Stacy, and du Vigneaud (13), who isolated and identified the compound. The present study confirms this finding and also demonstrates that α-ketobutyric acid is aminated to α-aminobutyric acid, probably by transamination, and that glutamic acid is probably the principal, if not the sole, amino donor in the transamination. The effects of additions of glutamic acid and α-ketoglutaric acid to dialyzed and non-dialyzed rat liver enzyme systems are presented in Table I. This and our previous finding that homoserine is metabolically formed from methionine explain the observation of Dent (15) of an increased ex-

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cretion in the urine of α-aminobutyric acid in a human subject following the oral administration of methionine. Such an explanation has been suggested previously by Carroll *et al.* (13).

No ammonia was formed when *O*-acetyl-DL-homoserine was incubated with a rat liver extract which converts free homoserine to α-ketobutyric acid and releases ammonia into the reaction mixture. This is analogous to the reported inactivity of serine and threonine dehydrases upon the derivatives of these β-hydroxyamino acids in which the β-hydroxyl hydrogen was substituted (16). It should be pointed out that the substitution of the hydroxyl hydrogen of homoserine with an acetyl group increases the

TABLE I

Distribution of Label of DL-*Homoserine-2-C*^{14} *in Incubation Products*

Incubation mixture, DL-homoserine-2-C^{14}, 1.7 μmoles (0.6 μc., 3.7×10^5 c.p.m.); rat liver extract, 5 ml. (see the text); and 20 μmoles of α-ketobutyric acid or L-glutamic acid, as indicated. The labeling of the metabolites is expressed in per cent of the total recovery of radioactivity, which represents 75 to 80 per cent of the starting radioactivity.

Product	Rat liver preparation			
	Non-dialyzed		Dialyzed	
	Control	Control + α-keto-*n*-butyric acid	Control	Control + glutamic acid
Propionic acid	1.2	0.6	4.0	1.1
α-Ketobutyric acid	1.7	16.6	14.8	0.2
α-Hydroxy-*n*-butyric acid	11.5	18.2	17.2	17.6
α-Amino-*n*-butyric acid	40.7	20.8	22.5	45.7
Homoserine	14.3	38.3	39.7	3.15

rate of oxidative deamination of homoserine by an ophio-L-amino acid oxidase.[1] This fact indicates that the acetyl substitution interferes with the conversion of homoserine to α-ketobutyric acid and suggests that dehydration is involved in the deamination mechanism.

The formation of α-hydroxybutyric acid can be explained by the action of certain dehydrogenases and the tendency toward equilibrium, in the incubation mixture, between α-ketobutyric acid and this compound. Thus,

[1] Unpublished observations of the authors with L-amino acid oxidase of moccasin venom (*Aghistrodon piscivorus*). DL-Homoserine is a very poor substrate for this enzyme. Substitution of the hydroxyl hydrogen by an acetyl group raised the Q_{O_2} value at 37° approximately 10-fold. (Q_{O_2} values for homoserine, *O*-acetylhomoserine, and leucine, determined on DL compounds and calculated for the L isomers of the enantiomorphs, are 13, 140, and 444, respectively.)

the L-amino acid oxidase of liver isolated by Blanchard *et al.* (17) has a higher activity on L-α-hydroxy acids than on L-amino acids. Also, lactic dehydrogenase of muscle has been reported to be active in reducing α-ketobutyric acid (18). We have observed that incubation of radioactive α-ketobutyric acid[2] with the rat liver extracts used in the present investigation yielded radioactive α-hydroxybutyric acid.

On the basis of the above findings we propose the metabolic pathway for homoserine, in the rat liver system studied, presented in Diagram 1. That α-ketobutyric acid is the first product in the pathway of metabolism was demonstrated by an experiment in which radioactive homoserine was incubated with an enzyme fraction precipitated between 33 to 66 per cent

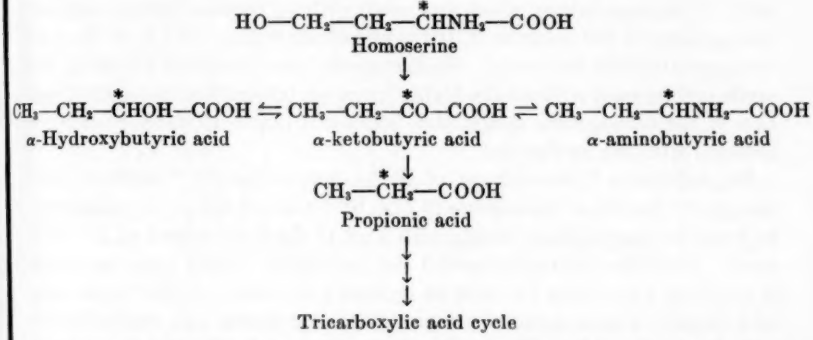

DIAGRAM 1. Sequence of reactions in the catabolism of homoserine-2-C^{14} illustrating the expected appearance of radioactive carbon-containing intermediates.

ammonium sulfate saturation. This fraction virtually yielded only radioactive α-ketobutyrate and no other radioactive products.

No significant amount of radioactivity was incorporated into homocysteine, methionine, threonine, or alanine. These compounds, if formed, should easily have been detected by our chromatographic procedure. Participation of homoserine in an oxidative deamination or a transamination reaction could not be demonstrated, because we could isolate neither radioactive α-keto-γ-hydroxybutyric nor α-keto butyrolactone from the reaction mixtures. Since no labeling of pyruvate, β-hydroxypropionate, or alanine occurred, metabolism of homoserine through α-keto-γ-hydroxybutyric acid does not seem to proceed to any significant extent under the conditions of the experiments. If α-keto-γ-hydroxybutyric acid were formed, it would be expected to give β-hydroxypropionic acid by decarboxylation, or to form

[2] Isolated by silica gel chromatography from incubation mixtures containing labeled homoserine.

formaldehyde and pyruvic acid, if the reverse of the reaction described by Hift and Mahler (19) is operative. Formation of radioactive pyruvic acid would, in turn, lead to the formation of radioactive alanine. This negative finding is in accord with the observation of Patwardhan (20).

EXPERIMENTAL

Preparation of Rat Liver Extract—Livers of adult, Long-Evans, male rats (300 to 350 gm.), fasted for 18 hours, were used in the experiments. The liver was homogenized in a Waring blendor at half speed for 1 minute with 2 volumes of cold 0.05 M phosphate buffer, pH 7.4. The homogenate was centrifuged at $300 \times g$ for 15 minutes to remove the coarse débris, and the supernatant liquid was centrifuged at $4 \times 10^4 \times g$ for 30 minutes in the cold. The supernatant liquid was used, with or without dialysis against two portions of 100 volumes of 0.01 M phosphate buffer, pH 7.4, in the cold for approximately 18 hours. Similar results were obtained by using the crude homogenate without the higher speed centrifugation, but centrifugation of the homogenate is desirable because it makes it easier to remove proteins after the incubation.

Incubations in Vitro—0.1 ml. of the DL-homoserine-2-C^{14} solution, containing 1.7 μmoles of homoserine (3.7×10^5 c.p.m. or 0.6 μc. of radioactivity) was incubated anaerobically with 5 ml. of the liver extract at 37° for 1 hour. After the incubation period the incubation vessels were immersed in a boiling water bath for 45 to 50 seconds and quickly chilled in running cold water. The coagulated proteins were centrifuged and washed twice with 2 ml. of water. The combined supernatant fluids were brought to pH 8 and dried *in vacuo* over calcium chloride and phosphorus pentoxide at room temperature. The dried material was redissolved in 0.5 ml. of water and acidified to pH 2 with 6 N HCl; 10 ml. of absolute ethanol were added to this solution, and the precipitate was removed by centrifugation. This procedure was repeated once, and the combined supernatant solutions were divided into two equal fractions and were dried *in vacuo* after adjusting the pH to 8. One of the processed incubation mixtures was assayed for the labeled nitrogenous compounds by chromatographing it on an acid Dowex 50 column (Fig. 2), and the other was chromatographed on a silica gel column to isolate non-nitrogenous carboxylic acids (Fig. 1).

Chromatography on Dowex 50-X8 Column—The procedure of Stein and Moore (21) was employed in this work. A resin bed of 0.9×110 cm. was used and the chromatograms were developed with 130 ml. of water, 200 ml. of 1.5 N HCl, and 250 ml. of 2.5 N HCl in the order mentioned. The flow rate was adjusted to 4 ml. per hour, and fractions were collected in 1 ml. volumes in polyethylene planchets and dried with infra-red lamps.

Chromatography on Silica Gel Column—Preparation of the silica gel column and construction of the solvent dispenser were done according to the

procedure of Kinnory, Takeda, and Greenberg (22). The benzene-ether and the chloroform-*tert*-amyl alcohol solvent systems described by these authors were used. The effluent was collected with a Technicon fraction collector in 80 drop fractions. This number of drops, under the particular set of experimental conditions, with the benzene-ether solvent system, gave the initial fraction volumes of 3 ml. The size of the fractions decreases as

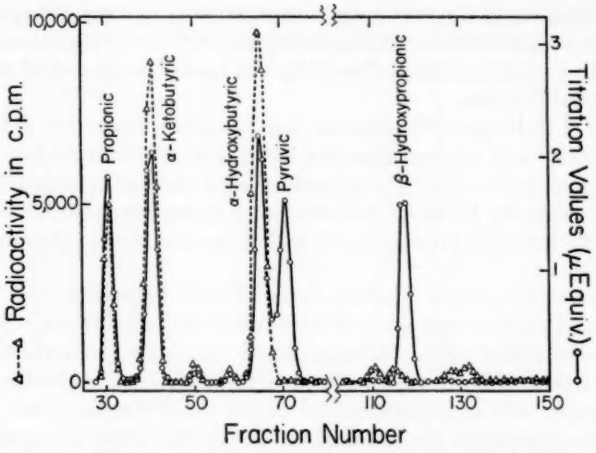

FIG. 1. Silica gel column chromatogram of the deproteinized extract obtained from the incubation of DL-homoserine-2-C^{14} with a dialyzed rat liver enzyme system, without the addition of glutamic acid (*cf.* Table I, fourth column). The benzene-ether solvent system was employed. Similar results are obtained with the chloroform-*tert*-amyl alcohol solvent system, except that a more complete separation of α-hydroxybutyric acid from pyruvic acid is effected by the latter. The minor peaks of radioactivity were not identified. Note that neither pyruvic acid nor β-hydroxypropionic acid is radioactive.

the concentration of ether in the solvent system increases during the course of the development of the chromatogram.

Standard chromatograms had been constructed previously with the two solvent systems by chromatographing authentic samples of compounds related to the present investigation singly and in combinations. It was learned that there is a striking parallelism between the order of elution of carboxylic acids in the benzene-ether mixture and their partition coefficients between ether and water. Collander gives the partition coefficients of about 300 organic compounds (23) in an ether-water system.

Approximately 5 μmoles of each of the known non-radioactive acids under study were added to the sample to be analyzed before chromatography in order to serve as carriers.

Further Identification of Intermediates—The radioactive intermediates of homoserine metabolism, isolated by column chromatography, were further identified as described below.

α-Ketobutyric Acid—Aliquots of the isolated α-ketobutyric acid were chromatographed on paper with an *n*-butanol-propionic acid mixture (95 parts of water-saturated *n*-butanol and 5 parts of propionic acid), together with aliquots of a standard preparation of the acid. The keto acid spot was revealed under ultraviolet light after spraying the chromatogram with 0.1 per cent semicarbazide hydrochloride and 0.15 per cent sodium acetate and heating at 110°. The radioactivity was located in the area of the ultraviolet-absorbing spot.

The 2,4-dinitrophenylhydrazone was prepared from the radioactive α-ketobutyric acid isolated from the incubation mixture and from an authentic preparation. Both preparations were chromatographed together on paper with the *tert*-amyl alcohol-ethanol-water mixture (5:1:4). Radioactivity was detected only on the yellow spots of the 2,4-dinitrophenylhydrazone.

Propionic and α-Hydroxybutyric Acids—These compounds were isolated by silica gel chromatography and were rechromatographed on paper with *n*-butanol saturated with 1.5 N ammonium hydroxide according to the technique of Reid and Lederer (24). Spots of the acids were detected by spraying the paper with an indicator solution, and the radioactivity was located on the chromatogram by counting sections of the paper chromatograms with a thin mica window counter. Precise correspondence of the radioactivity and the colored spots was obtained.

α-Aminobutyric Acid—α-Aminobutyric acid was isolated by Dowex 50 column chromatography and was rechromatographed on paper with three different solvents. In all cases the radioactivity coincided with the ninhydrin-positive spot of the α-aminobutyric acid. The three solvents used were phenol-water (80:20), *n*-butanol-acetic acid-water (3:2:1), and collidine-isoamyl alcohol-water (35:35:25).

Isolation of Alanine from Homoserine Incubations—In order to prove that alanine was not labeled, it was isolated from the homoserine incubations. As seen in Fig. 2, homoserine is eluted from the Dowex 50 column as an unusually wide peak, which overlaps with that of alanine.[3] By rechromatographing the combined fractions, which contained the alanine on a 0.9 × 55 cm. column of ammonium Dowex 50 with 0.2 M ammonium formate buffer, pH 3.1, containing 40 per cent ethanol, alanine was eluted at 110 to 130 ml. of the effluent, while homoserine was eluted at 75 to 88 ml.;[4] the alanine thus isolated had no detectable amount of radioactivity.

[3] Alanine is eluted from the Dowex 50 column at about the 265th fraction (see Fig. 2).

[4] A larger portion of homoserine was retained by the column in the form of α-amino-γ-butyrolactone.

Synthesis of DL-*Homoserine-2-C*^{14}

Synthesis—The lactone of homoserine-2-C^{14} was prepared by modifying the procedure of Painter (25).

To a solution prepared by warming 0.5 gm. of ethyl acetamidomalonate-2-C^{14} (3 μc. per mg.) in 5 ml. of absolute ethanol containing 0.056 gm. of sodium, 0.507 gm. of β-phenoxyethyl bromide and 12 mg. of dry potassium iodide were added. The mixture was refluxed for 24 hours and cooled, and an additional 0.250 gm. of phenoxyethyl bromide in 2 ml. of absolute ethanol containing 26 mg. of sodium was added. Refluxing was continued

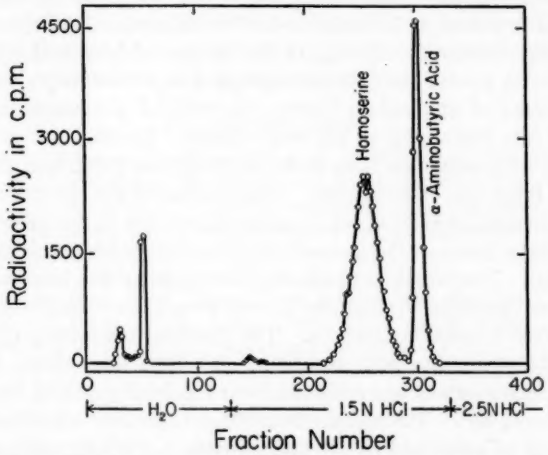

FIG. 2. Dowex 50-X8 column chromatogram of the deproteinized extract obtained from the incubation of DL-homoserine-2-C^{14} with a dialyzed rat liver enzyme system, without the addition of glutamic acid (*cf.* Table I, fourth column). The peaks of radioactivity eluted by water in the early fractions are due to carboxylic acids, most of which are lost by evaporation in drying the fractions.

for 18 hours; the solution was cooled, neutralized with acetic acid, and taken to dryness *in vacuo*. The residue was extracted repeatedly with dry ether. The ether was removed from the filtered extracts and the residue was saponified by refluxing with 2.5 ml. of 10 per cent NaOH for 2.5 hours. The resulting solution was distilled to dryness *in vacuo*, water was added, and the distillation was repeated. The residue was refluxed for 24 hours with 7 ml. of 6 N HCl and taken to dryness *in vacuo* three times, as above. The final residue was dissolved in water, treated with Norit, filtered, and concentrated to 3 ml. Pyridine was added to precipitate 280 mg. of *O*-phenylhomoserine-2-C^{14} (62 per cent). The mother liquors contained glycine-2-C^{14} and additional *O*-phenylhomoserine-2-C^{14} as shown by paper chromatograms.

The product was refluxed for 19 hours with 2.5 ml. of 48 per cent hydrobromic acid and evaporated to dryness three times as above. The residue was dissolved in water, treated with Norit, filtered, concentrated, and dried *in vacuo* over phosphorous pentoxide. The homoserine-2-C^{14} lactone hydrobromide contained approximately 0.6 per cent glycine-2-C^{14} and was purified finally as described below on a Dowex 50 exchange resin. The over-all yield of pure homoserine-2-C^{14} lactone, exclusive of the *O*-phenyl derivative remaining in the original mother liquors, was 38.2 per cent, based upon ethyl acetamidomalonate-2-C^{14}.

Purification—A chromatographic procedure described previously (14) was applied to isolate DL-homoserine-2-C^{14} from contaminating radioactive glycine. Approximately 150 mg. of the lactone of homoserine-2-C^{14} contaminated with glycine were chromatographed on a relatively short column (1.8 × 16 cm.) of ammonium Dowex 50, with 0.2 M ammonium formate buffer, pH 3.1, containing 40 per cent ethanol. Glycine was eluted from the column with some free homoserine in a discrete peak, and the lactone was eluted later, free from glycine. The portion of the chromatogram between the two peaks showed some radioactivity; this is due presumably to the equilibrium between the free acid and the lactone of homoserine in acid solution (26). The fractions containing both glycine and homoserine were combined and recycled through the Dowex 50 column to improve the yield of glycine-free homoserine lactone. The fractions containing glycine-free α-amino-γ-butyrolactone were combined and dried on a steam bath, and the ammonium formate was removed from the dried material by sublimation *in vacuo* at 35°. The residue from the sublimation was dissolved in a small amount of water and its pH was adjusted to 7.6 with sodium hydroxide. This solution was heated in a sealed Pyrex tube, together with 10 ml. of 15 M ammonium hydroxide at 110° for 4 hours. The ammonia was removed by drying the hydrolysate *in vacuo* over concentrated sulfuric acid.[5]

Purity of the final solution of DL-homoserine-2-C^{14} was tested by paper chromatography and radioautography.

Other Preparations—*O*-Acetyl-DL-homoserine was prepared according to the method employed by Sakami and Toennies (27) for the synthesis of *O*-acetyl derivatives of serine, threonine, tyrosine, and hydroxyproline. α-Ketobutyric and β-hydroxypropionic acids were kindly prepared by Mr.

[5] A paper chromatogram of an aliquot of the material obtained after the sublimation of the buffer salt, developed with *n*-butanol-acetic acid-water (3:2:1) as solvent, revealed four spots (R_F values, 0.20, 0.37, 0.41, and 0.52) of comparable radioactivities. Of these spots, two (R_F 0.20 and 0.52) were ninhydrin-positive. R_F values of homoserine and its lactone on the same chromatogram were 0.20 and 0.35, respectively. Upon hydrolysis with concentrated ammonium hydroxide only the spot of homoserine was detected on a paper chromatogram, and no other area of the chromatogram was radioactive.

D. C. Morrison of this laboratory. α-Hydroxybutyric acid was prepared in our laboratory by the action of potassium cyanide on the bisulfite addition product of propionaldehyde, followed by hydrolysis of the resulting cyanohydrin with hydrochloric acid.

SUMMARY

To establish the metabolic pattern of the main carbon chain of methionine, the enzymatically formed reaction products of DL-homoserine-2-C^{14} were determined upon incubation of the amino acid with rat liver homogenates. The radioactive products isolated and identified were α-ketobutyric, α-hydroxy-*n*-butyric, α-amino-*n*-butyric, and propionic acids. It is concluded that the primary initiating reaction in the catabolism of homoserine is the deamination to α-ketobutyric acid.

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KYNURENINE TRANSAMINASE FROM NEUROSPORA*

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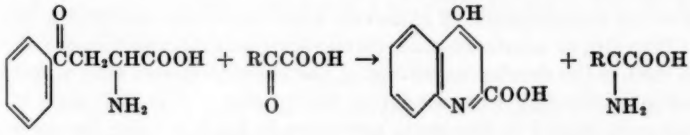
A study of the metabolism of kynurenine in *Neurospora crassa* has led to the investigation of the enzymes responsible for the formation of kynurenine (1) as well as of those forming intermediates in tryptophan and nicotinic acid biosynthesis (2, 3). The present communication describes another enzymatic reaction in which kynurenine participates. In the presence of an amino group acceptor kynurenine is deaminated and undergoes ring closure with the resulting formation of kynurenic acid.



That the latter half of the reaction, *i.e.* the ring closure of *o*-aminobenzoylpyruvic acid, may be non-enzymatic is suggested by the report of work on the oxidation of L-kynurenine by L-amino acid oxidase (4). The expected product of the reaction, *o*-aminobenzoylpyruvic acid, was not found while kynurenic acid accumulated.

Although kynurenic acid has not as yet been found to undergo further degradation nor been implicated in any physiological rôle, the ability to form this compound appears to enjoy a ubiquitous distribution. Thus, the transamination of kynurenine with concomitant formation of kynurenic acid has been reported in extracts of animal tissues (5, 6) as well as in *Pseudomonas fluorescens* (7, 8) and *Escherichia coli* (9).

The enzyme from *Neurospora*, kynurenine transaminase, has been found to utilize kynurenine and 3-hydroxykynurenine in the presence of pyridoxal phosphate and one of a variety of α-keto acids with the formation of kynurenic acid and xanthurenic acid, respectively.

* The work reported in this paper was supported in part by the Atomic Energy Commission (contract No. AT(30-1)-1017).

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Methods

The formation of kynurenic acid and xanthurenic acid was followed chromatographically. Kynurenic acid could be estimated visually on paper chromatograms by the intensity of its fluorescence following ultraviolet irradiation. No attempt was made to establish the quantity of xanthurenic acid formed.

Enzyme Assay—The assay for enzyme activity was conducted in a volume of 1 ml. which contained the following: 60 μmoles of phosphate buffer at pH 7.5, 12 mμmoles of pyridoxal phosphate, 25 μmoles of pyruvic acid, 5 μmoles of L-kynurenine (or 4 μmoles of 3-hydroxy-DL-kynurenine), and the enzyme preparation. The reaction was stopped after incubation for 1 hour at 37° with 0.2 ml. of a 10 per cent perchloric acid solution. After the removal of the protein precipitate by centrifugation, a 0.7 ml. aliquot of the supernatant solution was extracted with 0.5 ml. of water-saturated *n*-butanol. A 0.03 ml. aliquot of the butanol phase was applied to a sheet of Whatman No. 3 filter paper.

For the determination of kynurenic acid the solvent mixture of Mason and Berg (10) or *n*-butanol-water-formic acid (81:12:7, volume per volume) was used as the developing solvent. The chromatograms were allowed to develop in the dark with ascending solvent flow. The R_F values of the compounds related to this study are shown in Table I. For the detection of xanthurenic acid the solvent mixture of Mason and Berg (10) was used. Kynurenic acid was identified by its fluorescence under an ultraviolet light source after a previous exposure of 10 minutes to ultraviolet light. The fluorescence of xanthurenic acid was detected without previous exposure to ultraviolet light.

The amount of kynurenic acid formed was estimated by comparing the fluorescent spot produced by the unknown with the fluorescence of a series of spots of known kynurenic acid content which had been incubated with enzyme and chromatographed in a manner identical with that of the unknown. The method is most useful for the range of kynurenic acid concentration from 10 to 70 mμmoles per ml. of incubation mixture and can detect the formation of approximately 3 mμmoles of the compound in such a mixture.

As indicated in Table II, kynurenic acid formation is proportional to the enzyme concentration and to the time of incubation within the range used here.

Purification—Crude cell-free extracts of *N. crassa* may contain sufficient quantities of kynurenic acid to interfere with the transaminase assay when growth has taken place in a medium supplemented with tryptophan. Such preparations also have an active kynureninase present which will convert kynurenine to anthranilic acid. A partial purification of the kynurenine

transaminase system was therefore necessary before attempting to obtain quantitative data concerning it.

TABLE I

R_F Values of Kynurenine Metabolites

Compound	R_F	
	Solvent 1*	Solvent 2*
3-Hydroxy-DL-kynurenine	0.27	0.31
L-Kynurenine	0.47	0.37
N'-Formyl-L-kynurenine	0.48	0.32
Kynurenic acid	0.67	0.72
Xanthurenic acid	0.77	0.76
3-Hydroxyanthranilic acid	0.81	0.81
Anthranilic acid	0.93	0.87

* Solvent 1, solvent of Mason and Berg (10), methanol-*n*-butanol-benzene-water (2:1:1:1, volume per volume); Solvent 2, *n*-butanol-water-formic acid (81:12:7, volume per volume).

TABLE II

Proportionality of Kynurenic Acid Formation with Respect to Enzyme Concentration and Time of Incubation

Substrate, 5 μmoles of kynurenine.

Protein	Duration	Kynurenic acid formed
mg. per ml.	*min.*	*mμmoles*
0	60	0
0.77	60	15
1.54	60	30
2.31	60	45
2.31	0	0
2.31	20	15
2.31	40	30
2.31	60	45

Batches of 100 ml. of cell-free extract were treated with protamine sulfate and Norit in the same manner as previously described (1). The supernatant fluid from this treatment was fractionated with ammonium sulfate so that three fractions were obtained, each fraction being dissolved in 20 ml. of 0.1 M phosphate buffer at pH 7.5. The material precipitating between 0 and 45 per cent of saturation was discarded. The 45 to 60 per cent fraction contained approximately half of the kynurenine transaminase activity, but the bulk of the kynureninase also precipitated here. The 60

to 75 per cent fraction contained kynurenine transaminase and very little kynureninase. Refractionation of the 60 to 75 per cent fraction with ammonium sulfate (the fraction salting out between 55 to 75 per cent of saturation being retained) yielded a preparation which was free of kynureninase and which required pyridoxal phosphate for transaminase activity. Under standard assay conditions, approximately 200 γ of protein of the final preparation were required to produce 70 mμmoles of kynurenic acid. Because of the relatively large quantity of kynureninase present it was impossible to judge the activity of the crude extract or to estimate the yield of the final preparation.

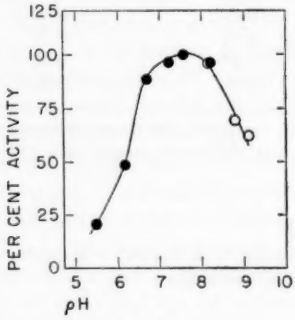

FIG. 1

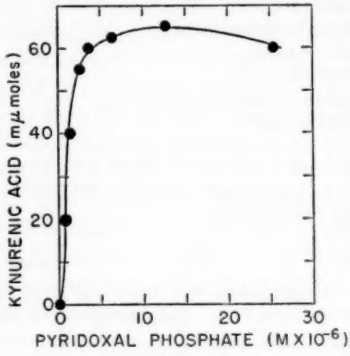

FIG. 2

FIG. 1. The effect of pH on transaminase activity; ●, 0.1 M phosphate; ○, 0.1 M tris(hydroxymethyl)aminomethane.

FIG. 2. The influence of pyridoxal phosphate concentration on kynurenic acid formation.

Results

Properties of Enzyme—The activity of kynurenine transaminase appears optimal at pH 7.5, as indicated in Fig. 1. Pyridoxal phosphate is required for the reaction, and this requirement cannot be replaced by pyridoxamine phosphate. The concentration of pyridoxal phosphate required for half maximal activity was found to be 1×10^{-6} M (Fig. 2).

Specificity for Amino Group Donor—L-Kynurenine was converted to kynurenic acid, the substrate concentration-activity curve being shown in Fig. 3. Half maximal activity was obtained at an L-kynurenine concentration of 1.5×10^{-3} M. It is to be noted, however, that, as the concentration of kynurenine is increased above 5×10^{-3} M, the transamination reaction is inhibited. As is the case with *Neurospora* kynureninase (11), kynurenine transaminase is inhibited by higher than optimal concentrations of both substrate and pyridoxal phosphate (Fig. 3, *A*, and Table III). Simi-

larly, the inhibition by either substrate or coenzyme appears to be reversed by coenzyme and substrate, respectively. D-Kynurenine is not utilized as a substrate for the enzyme.

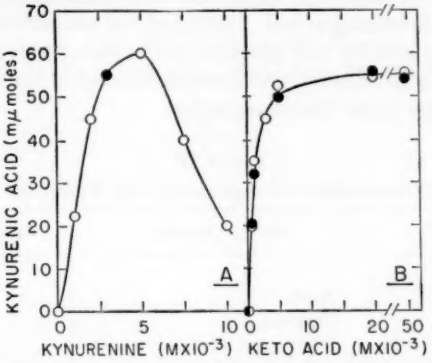

FIG. 3. Kynurenic acid formation as a function of (*A*) kynurenine concentration, and (*B*) keto acid concentration; ○, pyruvic acid; ●, α-ketoglutaric acid.

TABLE III

Inhibitory Effect of Higher Kynurenine and Pyridoxal Phosphate Concentrations

Kynurenine	Pyridoxal phosphate	Kynurenic acid formed
μmoles per ml.	*γ per ml.*	*mμmoles per ml.*
5	3.2	80
5	32	40
10	3.2	60
10	6.4	70
10	16	65
10	32	65
10	64	50

Although quantitative estimates of xanthurenic acid formation were not attempted, the substitution of 3-hydroxy-DL-kynurenine as amino donor permitted the formation of xanthurenic acid.

Specificity for Amino Group Acceptor—The effects of the various α-keto acids which were examined as amino group acceptors in the kynurenine transamination reaction are recorded in Table IV.[1] Most efficacious of these were pyruvic acid and α-ketoglutaric acid, while α-ketoisovaleric acid and *d*-α-keto-β-methylvaleric acid give rise to only traces of kynurenic acid. Phenylpyruvic acid, indolepyruvic acid, and α-ketobutyric acid were of an

[1] Generous quantities of the keto acids were the gifts of Dr. Alton Meister.

approximately equal activity which was slightly less than that of pyruvic and α-ketoglutaric acids. Half maximal activity was obtained (Fig. 3, *B*) at a pyruvate or α-ketoglutarate concentration of 7×10^{-4} M. In the case of pyruvic and α-ketoglutaric acids, the incubation mixture after deproteinization was chromatographed. Although not estimated quantitatively, the formation of alanine and glutamic acid, respectively, was observed. The presence of a glutamate-pyruvate transaminase was not demonstrable when examined by paper chromatography.

TABLE IV

Transamination of Kynurenine with Keto Acids

Experiment No.	Keto acid, 20 μmoles	Kynurenic acid formed
		mμmoles
1	None	0
	Pyruvic	55
	α-Ketoglutaric	55
	α-Ketobutyric	50
2	None	0
	Pyruvic	80
	Indolepyruvic	70
3	None	0
	α-Ketoglutaric	60
	Phenylpyruvic	55
	α-Ketoisovaleric	8
	d-α-Keto-β-methylvaleric	5

SUMMARY

An enzyme from *Neurospora crassa*, kynurenine transaminase, has been partially purified and found to transaminate kynurenine and 3-hydroxykynurenine to kynurenic acid and xanthurenic acid, respectively. A variety of α-keto acids acts as amino group acceptor; pyridoxal phosphate is required for enzyme action.

As is the case with *Neurospora* kynureninase, kynurenine transaminase is inhibited by high substrate and high pyridoxal phosphate concentration.

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THE REDUCTION OF L-XYLULOSE TO XYLITOL BY GUINEA PIG LIVER MITOCHONDRIA*

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(Received for publication, December 22, 1955)

There is a body of evidence suggesting that L-xylulose has a normal metabolic function. This pentose, which has long been known to be excreted in essential pentosuria, has recently been found in the urine of normal humans (2–4), guinea pigs (3), and rats (4). Furthermore, humans whose urine did not contain detectable amounts of the pentose excreted this sugar after ingesting large quantities of D-glucuronolactone (2, 3). There is also evidence that L-xylulose is metabolized by several species. The finding of little or no xylulose in urine led to the conclusion that it is metabolized by normal humans after oral administration (5) and by the mouse (2) and dog (6) after parenteral administration. A pentosuric human showed only a small increase in xylulose excretion after an oral dose (5). Increased urinary glucose indicated that depancreatized dogs metabolize both orally and intraperitoneally administered L-xylulose (7). Only in the rat has lack of utilization of the pentose been reported (8). The oral route of administration in the latter study may account for this exception, since L-xylulose is absorbed extremely slowly from the intestine of the rat (8).

In view of the abundant evidence that mammals can both produce and degrade L-xylulose, we investigated its metabolism in guinea pig liver. The guinea pig was chosen because it resembles man in having a dietary requirement for L-ascorbic acid. This vitamin is related to the pentose not only stereochemically but probably also in its biogenetic relationship to D-glucuronolactone (9, 10). In previous communications (1, 2) we reported that guinea pig liver slices, homogenates, and mitochondrial fractions caused the rapid disappearance of L-xylulose when incubated in the presence of required cofactors. The present paper presents enzymatic studies of this metabolic transformation, as well as chemical evidence that

* This study was supported by grants from the National Science Foundation and Eli Lilly and Company. A preliminary account was presented before the forty-sixth annual meeting of the American Society of Biological Chemists at San Francisco, April, 1955 (1).

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XUM

xylitol is the main product of the action of guinea pig liver mitochondria on L-xylulose.

During the course of our study there have been numerous reports of enzymatic reactions involving D-xylulose and its derivatives. It is now clear that the D-ketopentose occupies an essential position in the 6-phosphogluconate oxidation pathway of carbohydrate metabolism (11, 12).

EXPERIMENTAL

Materials and Methods

Materials—Most of the L-xylulose was isolated from pentosuric urine by the method of Greenwald (13) or by ion exchange chromatography of its borate complex (3). The pentose was also prepared by isomerization of L-xylose in boiling pyridine (14). After removal of most of the residual xylose by crystallization from alcohol-ether solution, the xylulose was purified by descending paper chromatography on Whatman No. 3 MM paper, with butanol-pyridine-water (10:3:3) as solvent (R_F xylulose, 0.38; R_F xylose, 0.28).

Several chemicals were generously contributed by Dr. N. K. Richtmyer (D-sorbose, D-gulitol, and D-talitol) and Dr. H. G. Fletcher (xylitol), both of the National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland, and by Dr. G. C. Mueller (L-erythrulose) of the University of Wisconsin. Dr. M. L. Wolfrom, of Ohio State University, kindly furnished seed crystals of xylitol pentaacetate. Other chemicals were preparations of highest purity available commercially.

Liver Preparations—Adult guinea pigs (400 to 600 gm.) of either sex were obtained from the Carworth Farms, Inc., New City, New York, and from the Empire Farms, Bainbridge, New York. The animals were always fasted at least 24 hours before being used for experiments. Liver slices were prepared with the Stadie tissue slicer and were tested in Krebs-Ringer-bicarbonate solution. Homogenates tested directly were prepared according to the procedure of Vestling *et al.* (15).

Mitochondria were prepared essentially according to a modification (16) of the differential centrifugation method of Hogeboom, Schneider, and Palade (17). The procedure was carried out at 0–5° and utilized the International PR-1 refrigerated centrifuge. Sufficient ice-cold 0.25 M sucrose was added to the minced liver of one animal to yield 50 to 60 ml. of suspension. Homogenization of the liver was effected in a manually operated Potter-Elvehjem glass homogenizer for 10 to 20 minutes or with an electrically driven Teflon pestle in a glass tube for 3 to 5 minutes. The suspension was brought to a volume of 100 ml. with 0.25 M sucrose, and then the "nuclear" fraction (containing nuclei, cell débris, unbroken cells, and blood cells) was collected by two centrifugations of the homogenate at 2000

r.p.m. for 5 minutes. To 90 ml. of the supernatant fluid were added dropwise 10 ml. of 1.5 M KCl. After 10 minutes, the mitochondria were collected by centrifugation at 3400 r.p.m. for 20 minutes, the supernatant fluid being reserved for separation of the microsomes. The mitochondrial precipitate was resuspended in 90 ml. of 0.15 M KCl-0.01 M $NaHCO_3$ and collected by centrifugation at 3400 r.p.m. for 10 minutes. The washing was repeated with 60 ml. of 0.15 M KCl-0.01 M $NaHCO_3$. The mitochondria were stored in KCl-$NaHCO_3$ at 0° until used or were suspended immediately in an amount of 0.154 M phosphate buffer (pH 7.5) to yield a concentration approximately equivalent to a "50 per cent" whole liver homogenate. (Thus, 0.3 ml. of this suspension would contain the mitochondria derived from 0.15 gm. of liver.) The washing procedure removed essentially all of the sucrose used in the homogenization, as shown by negligible ketose tests given by the final mitochondrial suspensions.

The sucrose supernatant fluid remaining after sedimentation of the mitochondria was centrifuged for 1 hour at maximal speed (about 19,000 r.p.m.) in the high speed attachment of the International refrigerated centrifuge. The microsome deposit from 10 to 20 gm. of liver was resuspended in 50 ml. of 0.15 M KCl-0.01 M $NaHCO_3$ and centrifuged again for 1 hour at maximal speed. The microsomes were then suspended in phosphate buffer, as were the mitochondria.

The tests of liver fractions were made in 25 ml. stoppered Erlenmeyer flasks shaken mechanically at 37°. Cofactor solutions were adjusted to pH 7.5, or the final solutions were adjusted to this pH before incubation.

Analytical Methods—Incubation mixtures were deproteinized by the $ZnSO_4$-$Ba(OH)_2$ procedure (15) or with 9 volumes of 0.55 M trichloroacetic acid. The following analytical reactions were employed: for ketopentose, the Dische-Borenfreund cysteine-carbazole reaction (18) and the Lampen modification (19) of the orcinol reaction; for reducing sugar, the Nelson method (20); for hexose, the anthrone method (21). Analysis of periodate-reacting material in incubation mixtures was carried out as follows: To a deproteinized aliquot containing at least 0.2 mg. of xylulose and pentitol were added 4.0 ml. of 0.015 M $NaIO_4$ and 2.0 ml. of 5 per cent $NaHCO_3$. After 1.5 hours at room temperature, buffer and potassium iodide were added, and the titration was completed with thiosulfate according to the method of Voris *et al.* (22). Periodate-reacting substances were detected on paper chromatograms by the method of Metzenberg and Mitchell (23).

Results

Experiments with Liver Slices and Homogenates—Guinea pig liver slices in Krebs-Ringer-bicarbonate solution, with 95 per cent O_2-5 per cent CO_2 as the gas phase, were found to cause the disappearance of L-xylulose

and D-fructose at equal rates. Since liver contains a very active fructokinase (15, 24), these results encouraged further study of the system responsible for the utilization of the pentose. The method of Vestling *et al.* (15)

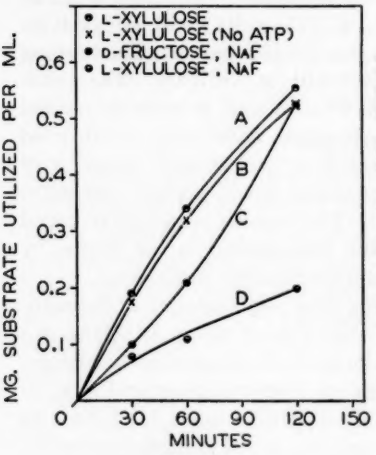

FIG. 1

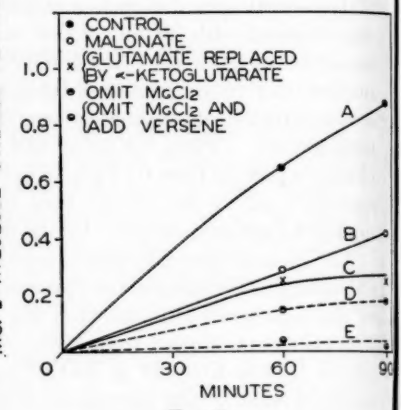

FIG. 2

FIG. 1. Utilization of L-xylulose and D-fructose by guinea pig liver homogenates. Each flask contained 0.6 ml. of 0.5 M glutamate, 0.6 ml. of 0.154 M phosphate buffer at pH 7.5, 0.6 ml. of 0.08 M $MgCl_2$, 1.2 ml. of "20 per cent" liver homogenate, and 6.0 mg. of substrate. Total volume, 6.0 ml. Flasks A, C, and D also contained 0.6 ml. of 0.05 M Na_2ATP. Flasks C and D contained NaF in a concentration of 0.05 M. Analysis of Ba-Zn filtrates by cysteine-carbazole method.

FIG. 2. Effect of malonate, α-ketoglutarate, and magnesium chloride on utilization of L-xylulose by guinea pig liver homogenates. Each flask contained 0.6 ml. of 0.154 M phosphate buffer at pH 7.5, 0.6 ml. of 0.05 M Na_2ATP, 1.2 ml. of "20 per cent" liver homogenate, and 6.0 mg. of xylulose. Total volume, 6.0 ml. Other components were Flask A, 0.6 ml. of 0.08 M $MgCl_2$, 0.6 ml. of 0.5 M glutamate; Flask B, same as A and 0.01 M to malonate; Flask C, 0.6 ml. of 0.08 M $MgCl_2$, 0.6 ml. of 0.5 M α-ketoglutarate; Flask D, 0.6 ml. of 0.5 M glutamate; Flask E, 0.6 ml. of 0.5 M glutamate and 0.0005 M to Versene. Analysis of Ba-Zn filtrates by cysteine-carbazole method. (Broken lines for Curves D and E signify only that they were obtained from a different experiment from the other curves. Plotting on one graph was permissible because the control flask in each experiment showed similar xylulose disappearance.)

for preparing and testing rat liver homogenates for fructokinase activity was chosen for study of the xylulose system. Homogenates of guinea pig liver in phosphate buffer containing L-glutamate, magnesium chloride, and adenosine triphosphate, again with 95 per cent O_2-5 per cent CO_2 as the gas phase, utilized L-xylulose as rapidly as D-fructose. Unlike fructose, however, xylulose utilization was considerably inhibited by the presence of

0.05 M fluoride and did not require the presence of adenosine triphosphate (ATP) in the medium (Fig. 1). In Fig. 2 it is seen that 0.01 M malonate is inhibitory, that α-ketoglutarate is less effective than glutamate, and that omission of $MgCl_2$ markedly decreases the disappearance of the pentose.

Replacement of oxygen by a nitrogen atmosphere was inhibitory, unless ATP was present in the medium (Fig. 3). Although this finding might suggest that phosphorylation of the pentose occurred, no direct evidence for such a reaction was obtained. In this experiment, and in most others involving liver homogenates, both barium-zinc and trichloroacetic acid filtrates were analyzed by the cysteine-carbazole and the orcinol reactions. Tests for hexoses and reducing sugars were also performed. In no case did analyses suggest the formation of any free or phosphorylated sugar as a result of the metabolism of L-xylulose. This held also when 0.01 M malonate or 0.005 M iodoacetate was used to inhibit the transformation. Manometric experiments, with the medium containing glutamate, magnesium chloride, and phosphate buffer, showed that xylulose disappearance was not accompanied by increased oxygen consumption or carbon dioxide evolution.

Among the other observations made with homogenates may be mentioned: (1) considerable decrease in enzymatic activity upon storage of the homogenate at 2° for 24 hours or upon dialysis against phosphate buffer at 5° for 3 hours, (2) failure of diphosphopyridine nucleotide (DPN) to restore activity to dialyzed homogenates, (3) inability of α-ketoglutarate and DPN together to replace glutamate, and (4) decreased activity when Veronal buffer was used in place of phosphate in the preparation of homogenate (with subsequent addition of phosphate for testing of enzymatic activity). Homogenates of other tissues were tested, but only the kidney showed activity comparable to that of liver.[1] Hamster and rat liver were less active than guinea pig liver. (In most homogenate experiments, ATP and 95 per cent O_2-5 per cent CO_2 were both used to insure optimal conditions.)

The testing of glucuronolactone as an inhibitor of xylulose utilization was suggested by the effect of this substance in enhancing xylulose excretion in pentosuric and normal humans and in guinea pigs (2, 3, 25). It will be recalled that aminopyrine and glucuronogenic substances also markedly enhance L-xylulose excretion by pentosuric individuals (25). In Fig. 4 it is seen that glucuronolactone, aminopyrine, and ammonium menthyl glucuronide inhibit xylulose utilization. The mechanism of action of these substances was not determined, as it appeared more desirable to seek purer enzyme preparations by studying the activity of liver fractions.

Experiments with Mitochondrial Preparations—A preliminary experi-

[1] Experiment performed by Mr. B. M. Silbert.

ment demonstrated that essentially all of the activity of a homogenate was sedimentable by centrifugation at 4000 r.p.m. for 1 hour at 0° (Fig. 5).[2] Differential centrifugation showed that the mitochondria contained the en-

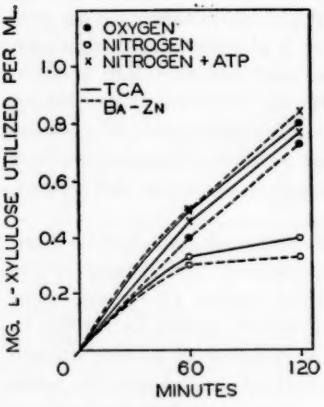

FIG. 3

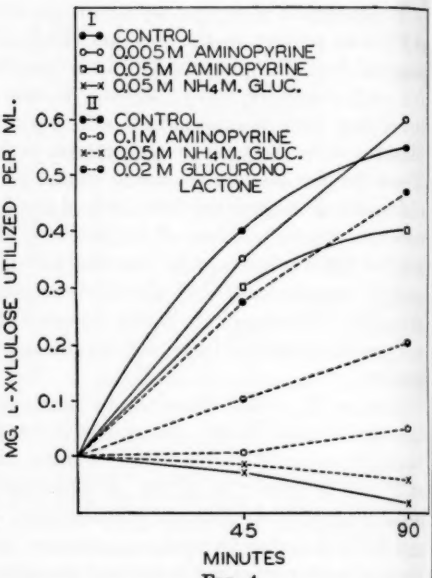

FIG. 4

FIG. 3. Effect of O_2 and N_2 on utilization of L-xylulose by guinea pig liver homogenates. Each flask contained 0.6 ml. of 0.5 M glutamate, 0.6 ml. of 0.154 M phosphate buffer at pH 7.5, 0.6 ml. of 0.08 M $MgCl_2$, 1.2 ml. of "20 per cent" liver homogenate, and 6.0 mg. of xylulose. Total volume, 6.0 ml. Analysis of Ba-Zn and trichloroacetic acid filtrates by cysteine-carbazole method.

FIG. 4. Effect of D-glucuronolactone, aminopyrine, and ammonium menthyl glucuronide on utilization of L-xylulose by guinea pig liver homogenates. Each flask contained 0.6 ml. of 0.5 M glutamate, 0.6 ml. of 0.154 M phosphate buffer at pH 7.5, 0.6 ml. of 0.08 M $MgCl_2$, 1.2 ml. of "20 per cent" liver homogenate, and 7.2 mg. of xylulose. Total volume, 6.0 ml. Analysis of Ba-Zn filtrates by cysteine-carbazole method.

zymatic system responsible for the disappearance of L-xylulose, the nuclear fraction and the microsomes having negligible activity (Table I). As with the whole homogenates, the mitochondrial activity is somewhat inhibited by aminopyrine and by fluoride and completely inhibited by iodoacetate (Fig. 6). Mitochondria have shown a variable requirement for ATP. In

[2] Useful consultations with Dr. Jane Harting Park and Dr. W. C. Holland regarding cell fractionation are gratefully acknowledged.

early experiments there was little disappearance of xylulose, even in the presence of oxygen, if ATP was not added to the medium. In later experiments consistently high utilization occurred in the absence of added ATP, even with nitrogen as the gas phase. The cause of this change has not yet been determined, but it is possible that it is due to the known sensitivity of mitochondrial transformations to brief exposure of the particles to low concentrations of various ions such as phosphate (26). It is hoped

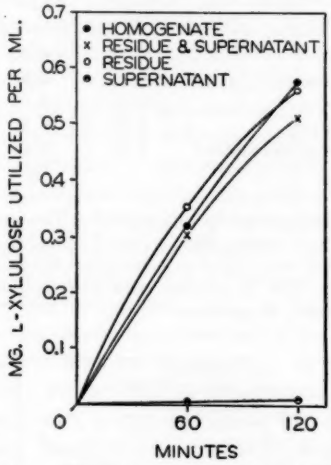

FIG. 5. Utilization of L-xylulose by readily sedimentable fraction of guinea pig liver homogenate. Each flask contained 0.4 ml. of 0.5 M glutamate, 0.4 ml. of 0.154 M phosphate buffer at pH 7.5, 0.4 ml. of 0.08 M $MgCl_2$, 0.4 ml. of 0.05 M Na_2ATP, 4.8 mg. of xylulose and 0.8 ml. of liver fractions equivalent to "20 per cent" homogenates. Total volume, 4.0 ml. Analysis of Ba-Zn filtrates by cysteine-carbazole method.

that experiments in progress will disclose the cause of variation in ATP requirement.

Mitochondria lose little activity when stored in 0.15 M KCl-0.01 M $NaHCO_3$ for 2 days at 0°. Attempts to liberate the enzyme by freezing and thawing of mitochondria at various temperatures and in the presence of various cofactors always led to inactivation. Among the cofactors used, in addition to the usual components of incubation mixtures, were glutathione, thiamine pyrophosphate, and DPN. The addition of these together with triphosphopyridine nucleotide (TPN), coenzyme A, and uridine triphosphate did not enable acetone powders prepared from mitochondria to utilize L-xylulose (nor did these substances increase the activity of intact mitochondria). The use of deoxycholate for the libera-

tion of the enzyme was precluded by the fact that addition of this agent to the medium in a concentration of 1 per cent caused complete inactivation.

TABLE I

Utilization of L-*Xylulose by Particulate Fractions of Guinea Pig Liver**

Experiment No.	Particulate fraction	TCA filtrate		Ba-Zn filtrate	
		Cysteine-carbazole	Orcinol	Cysteine-carbazole	Orcinol
5	Nuclear	0.13	0.00	0.22	0.00
	Mitochondrial	0.71	0.59	0.60	0.53
7	Nuclear	0.01	−0.15	0.09	−0.14
	Mitochondrial	0.66	0.53	0.76	0.41
	Nuclear + mitochondrial	0.48	0.45	0.45	0.32
	Microsomal			−0.05	

* The disappearance of xylulose in mg. per ml. The incubation medium contained glutamate, phosphate buffer, $MgCl_2$, Na_2ATP, and xylulose as in Fig. 5, except that the liver fractions were added as suspensions equivalent to a "50 per cent" whole liver homogenate. The incubation time with microsomes was 60 minutes and with all other fractions, 90 minutes.

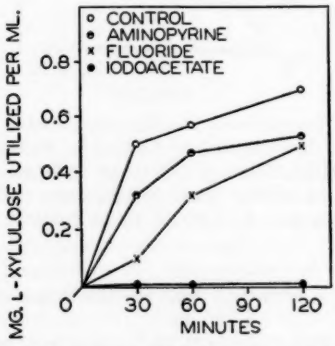

FIG. 6. Inhibition of mitochondrial utilization of L-xylulose by aminopyrine, fluoride, and iodoacetate. Flask contents the same as in Table I. The final concentrations of inhibitors were 0.07 M aminopyrine, 0.05 M fluoride, and 0.005 M iodoacetate. Analysis of Ba-Zn filtrates by cysteine-carbazole method.

Rupture of mitochondria without significant loss of activity was finally accomplished by allowing them to stand for 1 hour at 0° in a volume of water calculated to yield a suspension equivalent to a "50 per cent" liver homogenate. To insure disruption of the remaining unruptured swollen particles, the solution was then vigorously homogenized in a Potter-Elvehjem glass

homogenizer. Efficiency of the rupture of the particles was confirmed by phase microscopy. Essentially all of the activity of intact mitochondria was found in the precipitate obtained by centrifugation of the mitochondrial homogenate at 3400 r.p.m. for 10 minutes. Unlike intact mitochondria, however, the activity of this mitochondrial residue is lost after 24 hours at 0°. This fraction is being studied at present in an attempt to learn the mechanism of the enzymatic reaction and to extract the enzyme system.

Preliminary experiments indicate that xylulose disappearance is dependent upon the formation of reduced DPN. If the mitochondrial residue is washed twice with distilled water, no activity is found in the presence of the usual cofactors. On the assumption that the washing process removed the soluble glutamic dehydrogenase remaining with the precipitate, the washed residue was tested with reduced diphosphopyridine nucleotide (DPNH) in place of glutamate. Disappearance of xylulose occurred.[3]

Isolation and Identification of Xylitol—As indicated earlier in this paper, various analytical approaches failed to yield suggestive evidence regarding the nature of the xylulose product. Since it was possible that a polyol might be the reaction product, study of the consumption of periodate by incubation mixtures was undertaken. It was found that xylulose disappearance was not accompanied by a decrease in periodate utilization, a result consistent with the formation of a polyol. Analysis by paper chromatography showed that, as the xylulose disappeared, there appeared a new substance which responded to periodate spray but not to any of the usual color tests for sugars. It had the same mobility as xylitol and arabitol (the two possible reduction products of xylulose) in a variety of solvents.

The pentitol was isolated from the reaction mixture as follows: 70 mg. of L-xylulose (which had been purified through the crystalline *p*-bromophenylhydrazone derivative) were incubated at 37° with 7.0 ml. of 0.08 M magnesium chloride, 7.0 ml. of 0.5 M glutamate, 7.0 ml. of 0.05 M ATP, 7.0 ml. of 0.154 M phosphate buffer (pH 7.5), and 18 ml. of "50 per cent" mitochondrial suspension in 0.154 M phosphate buffer, the volume being made up to 70 ml. with water. Analysis of an aliquot by the cysteine-carbazole reac-

[3] The enzyme system has recently been rendered soluble by treatment of the mitochondrial residue with butanol. The L-xylulose-xylitol interconversion catalyzed by the extract proceeds much more rapidly with triphosphopyridine nucleotide than with DPN, indicating that the enzyme is unique among polyol dehydrogenases thus far reported, all of which are DPN-dependent. The extract contains a second enzyme which catalyzes the interconversion of xylitol and D-xylulose. This enzyme is DPN-dependent and seems to be less specific in substrate requirement than the L-xylulose enzyme. The two dehydrogenases make possible the biological conversion of L-xylulose to D-xylulose and therefore may provide a route for the L-ketopentose to be metabolized via the 6-phosphogluconate pathway (Hollmann, S., and Touster, O., unpublished results).

tion (after Ba-Zn deproteinization) indicated 60 per cent utilization of the xylulose in 105 minutes. The remaining incubation mixture was deproteinized by heating in a boiling water bath for 3 minutes and then filtering the solution. The filtrate was deionized over 1 × 4 cm. columns of Amberlite IR-120 and of Duolite A-4 and then evaporated to dryness *in vacuo*. The residual oil was extracted with absolute methanol, the resulting solution being applied to Whatman No. 3 MM paper for isolation of the pentitol. The solvent for the descending chromatographic separation was 80 per cent propanol. The pentitol was located by spraying a narrow strip of the dried paper with periodate. It was eluted with water from the corresponding area of the unsprayed paper. The eluate (10 to 12 ml.) was evaporated to dryness, and the residual oil was extracted with absolute methanol. The extract was clarified by centrifugation and evaporated to dryness. An aqueous solution (1 to 2 ml.) of the residue was decolorized over a charcoal-Celite column and evaporated to dryness, yielding 31 mg. of oil which could not be crystallized, although it appeared to be homogeneous by paper chromatography. A sample of it utilized only 50 per cent of the periodate required on the assumption that it was a pure pentitol. Since all paper chromatographic analyses indicated that the only periodate-reacting material present was a pentitol, the product was converted into an acetyl derivative.

To 29 mg. of the oil were added 0.3 ml. of acetic anhydride and 0.3 ml. of pyridine, and the resulting mixture was shaken mechanically at room temperature for 7 hours. Since dilution of the reaction mixture with water caused the formation of only a small amount of precipitate (xylitol pentaacetate precipitated at this point when pure xylitol was acetylated), the diluted mixture was evaporated to dryness *in vacuo*. The residual oil was dried by four additions, with subsequent distillation, of several ml. portions of benzene. The residue was then dissolved in 1 ml. of benzene and 2 ml. of ligroin, a trace of insoluble material being removed by centrifugation. Upon standing 1 day in the freezer the solution yielded large crystals which were collected by centrifugation and washed twice with ligroin. This product weighed 15.4 mg. and melted at 56–60°,[4] with a little softening at 50°. That it was slightly impure xylitol pentaacetate was indicated by the finding that a mixture with authentic xylitol pentaacetate (m.p. 61°) melted at 53–58°, again with a little softening at 50°. Recrystallization from benzene-ligroin improved the melting point only slightly, so that a final recrystallization was carried out in 16 per cent ethanol. The crystalline product which formed during several days in the refrigerator was washed with water and dried. It melted at 60–61° and did not depress the melting point of xylitol pentaacetate (arabitol pentaacetate melts at 78°). Infra-red analy-

[4] All melting points were taken on the Fischer-Johns micro block.

sis[5] confirmed the structure of the derivative in that it had a spectrum identical with that of xylitol pentaacetate and differed greatly from that of arabitol pentaacetate. Xylitol was also isolated as the pentaacetate in two other mitochondrial experiments.

Reversibility and Specificity of Reaction—When xylitol is substituted for L-xylulose in the customary incubation medium containing mitochondria, a small amount of the ketopentose is formed, as indicated by the cysteine-carbazole reaction. Omission of glutamate from the medium is without effect, but inclusion of methylene blue increases the amount of xylulose formed. No substrates other than L-xylulose and xylitol have been found. The substances tested were D-xylulose, D-sorbose, L-sorbose, L-erythrulose, D-fructose, D-sorbitol, L-arabitol, D-gulitol, and D-talitol. The polyols were studied in the presence of methylene blue. Ketose formation or disappearance was determined by the cysteine-carbazole reaction except in the case of L-erythrulose, when it was necessary to measure reducing power. The sugars and polyols were in general chosen with a view to establishing whether the mitochondrial enzyme resembled known enzymes which catalyze the interconversion of ketoses and polyols. While other substances will be tested, the evidence to date indicates that the mitochondrial system is specific for the L-xylulose-xylitol transformation.

DISCUSSION

The occurrence of L-xylulose in small amounts in normal urine and in much larger quantities in pentosuric urine should encourage further studies directed toward elucidating reactions which may be responsible for both the formation and disposition of the pentose. Acceptance of the enzymatic reduction of L-xylulose, or the oxidation of xylitol, as a normal metabolic reaction will depend in part upon the discovery of other transformations involving these substances. Although in none of our mitochondrial experiments with L-xylulose as substrate was any evidence obtained for the accumulation of a product other than xylitol, it is possible that this substance is further metabolized in a tissue other than liver or even in another cellular fraction of the liver. An analogy for the latter possibility is the biosynthesis of glucuronides, which involves uridine diphosphate glucuronic acid formation in the soluble fraction of liver, followed by transfer of the glucuronic acid moiety to an aglycone in the microsomes (27). Guinea pig liver contains a soluble enzyme which converts xylitol to D-xylulose (28). Since the latter substance is a substrate of yeast transketolase (29), a possible route may be open for the metabolism of L-xylulose through established pathways.

[5] We are greatly indebted to Dr. J. R. Lawson, Tennessee Agricultural and Industrial State University, for the infra-red analyses.

Comparison of the enzyme of guinea pig liver mitochondria with other "ketose reductases" which have been reported discloses marked differences in substrate specificity. The soluble rat liver enzyme of Blakley (30), originally discovered because of its action on D-fructose and D-sorbitol, also utilizes a number of other ketoses and polyols as substrates (28), with D-xylulose being produced from xylitol. Williams-Ashman and Banks (31) have reported the occurrence of the same enzyme in the seminal vesicle and coagulating gland of the rat, but comparison of their report with that of McCorkindale and Edson (28) shows that the enzyme of the former workers differed in being able to catalyze the dehydrogenation of L-arabitol. Shaw (32) has described an enzyme, from an organism tentatively identified as a species of *Pseudomonas*, which interconverts xylitol and L-xylulose with DPN as coenzyme. This enzyme also showed high activity with D-gulitol and D-talitol, neither of which is oxidized by the liver mitochondrial system described in the present paper.

It is apparent that guinea pig liver contains a highly specific enzyme for the reduction of L-xylulose. In the absence of substrates other than L-xylulose and xylitol, the transformation may be regarded as a normal metabolic reaction.

SUMMARY

Guinea pig liver mitochondria catalyze the reduction of L-xylulose to xylitol, which was identified as its crystalline pentaacetyl derivative. Several inhibitors of the reaction were found. Reversibility of the reaction was indicated by formation of ketopentose from xylitol. The enzyme system is located in the insoluble portion of the mitochondria and appears to involve pyridine nucleotides.

The high order of specificity of the system was evident from its lack of activity toward all ketoses and polyols tested other than L-xylulose and xylitol. The substrate specificity differentiates the mitochondrial enzyme from other enzymes which are known to catalyze the interconversion of ketoses and polyols.

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THE QUANTITATIVE DETERMINATION OF ACETOL*

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The results of studies in this and other laboratories suggest that acetol is an intermediate in the metabolism of acetone, 1,2-propanediol, lactic acid, and pyruvic acid (1–6). In order to pursue our interest in this metabolite, it was necessary to develop a sensitive, specific, and quantitative method for acetol. Forist and Speck (7) and Werle and Stiess (8) reported that acetol will condense with *o*-aminobenzaldehyde in an alkaline solution to form 3-hydroxyquinaldine, which can be measured fluorometrically. This condensation reaction is based upon the original observation of Baudisch and Deuel (9), who believed it to be specific for acetol. Furfural, diacetyl, formaldehyde, and monosaccharides, especially glucose, interfere in this determination when the condensation reaction is carried out at 100° in a strongly alkaline solution (7, 8). We have found that this reaction can be used successfully by modifying the conditions.

Acetol is readily condensed with *o*-aminobenzaldehyde in an alkaline solution while being shaken for 1 hour at room temperature and is quantitatively determined fluorophotometrically by a direct method. An indirect method for the determination of acetol is proposed which is based on observations that acetol, treated with copper-lime (10), is converted to lactic acid, which in turn is determined colorimetrically. A comparison of these two methods is made.

EXPERIMENTAL

Materials

A 1 per cent solution of *o*-aminobenzaldehyde[1] in methanol was diluted 1:50 with water just before being used. A standard solution of acetol[1]

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[1] *o*-Aminobenzaldehyde was obtained from the Delta Chemical Works, New York, and the acetate ester of acetol was obtained from the Eastman Kodak Division of Distillation Products, Inc.

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was prepared and standardized by iodometric titration (11, 12) and also by oxidation to formaldehyde with periodate, followed by estimation of the formaldehyde with chromotropic acid (13). A solution containing 4 μmoles per ml. of ethylenediaminetetraacetic acid was prepared. The reagents for the determination of lactic acid were prepared as previously described (10). A Coleman junior spectrophotometer was used for the colorimetric analysis and a Farrand fluorometer or Coleman electronic photofluorometer for the fluorometric analysis.

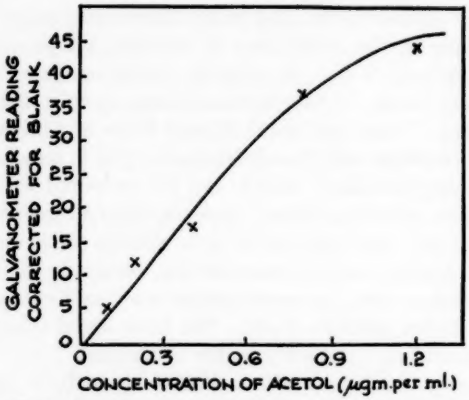

FIG. 1. The effect of varying concentrations of acetol on the production of fluorescence with *o*-aminobenzaldehyde. The corrected galvanometer readings are plotted against the concentration of acetol in the reaction mixture after the final dilution is made.

Methods

Direct, Fluorometric Method for Determination of Acetol—Duplicate aliquots of a solution containing no more than 0.4 μmole (25 to 30 γ) of acetol were added to test-tubes, graduated at the 10 ml. mark, and were diluted with water to make a volume of 4 ml. 0.5 ml. of ethylenediaminetetraacetic acid solution was added to each tube. 1 ml. of *o*-aminobenzaldehyde solution was added to one tube, and to the other 1 ml. of water to serve as the blank for that sample. The contents of each tube were mixed; then 1.25 ml. of sodium hydroxide (0.52 N) were added and mixed. The tubes were shaken for 1 hour at room temperature in a mechanical shaking machine, after which each tube was acidified with 1.5 ml. of hydrochloric acid (1 N). Subsequently, an excess of solid sodium bicarbonate was added which brought the pH to approximately 8. All the tubes were diluted to 10 ml. with water. If the concentration of acetol was too high, it was sometimes necessary to make a 1:2 dilution of the reaction mixture with

water in order to measure the fluorescence on the Farrand fluorometer. A primary filter (Corning No. 5960) and a secondary filter which consisted of a combination of Corning filters, Nos. 3389 and 4308, were used. Filter No. 3389 of the secondary filter was placed toward the sample. The sensitivity of the instrument was adjusted by setting the galvanometer at 40, a standard solution of 0.02 γ of quinine sulfate per ml. in 0.1 N sulfuric acid being used. A Coleman electronic photofluorometer may be used with filters B-1 and P-C-1 as the primary and secondary filters, respectively, but it is not as sensitive as the Farrand fluorometer. The Coleman fluorometer was adjusted by setting the galvonometer at 85, a standard solution of 0.3 γ of quinine sulfate per ml. in 0.1 N sulfuric acid being used.

The data presented in Fig. 1 were obtained when acetol, in concentrations up to 2.4 γ per ml. of reaction mixture, was condensed as described with *o*-aminobenzaldehyde and diluted 1:2 before the concentration of the fluorescing compound was determined.

The results indicate that the fluorescing product is stable at room temperature for 7 hours and only 25 per cent is lost after 18 hours, at room temperature, which is in agreement with data reported by Forist and Speck (7).

Indirect, Colorimetric Method for Determination of Acetol—It has been reported that acetol is converted to lactic acid by an alkaline solution (sodium hydroxide) of copper sulfate and potassium sodium tartrate, Fehling's solution (14). The copper-lime purification treatment in the method for lactic acid (10) is an alkaline solution (calcium hydroxide) of copper sulfate, and, therefore, it seemed probable that acetol would be converted to lactic acid by the copper-lime treatment.

A tissue extract containing acetol, or a standard solution of acetol, was analyzed for lactic acid (10). This determination was carried out after a 6 hour treatment with phosphatase (15) and the values obtained were corrected for 1,2-propanediol contribution. The value thus obtained was called "total corrected lactic acid." It has been found that 1 mole of 1,2-propanediol is equivalent to 0.17 mole of lactic acid under the experimental conditions used in this laboratory. An aliquot of the same extract as well as the standard solution of acetol was oxidized with periodate and aerated with nitrogen, as in the procedure for the determination of 1,2-propanediol (16). Acetol under these conditions is converted to acetic acid and formaldehyde (17). Iodate and periodate ions interfere in the lactic acid determination and therefore the oxidation mixture, 7.5 ml. (16), was treated with 2.5 ml. of 20 per cent lead subacetate solution to precipitate these ions. After centrifugation, the supernatant fluid A was made slightly acidic, pH 4 to 5, with hydrochloric acid (5 N, 2 drops) and dimedon was added to precipitate the formaldehyde which interferes in the

determination of lactic acid (18). The formaldehyde-dimedon complex was removed by centrifugation, 5 ml. of the supernatant fluid B were purified by the copper-lime procedure, and lactic acid was determined. 1 ml. of 20 per cent lead-subacetate solution was carried through the copper-lime treatment to serve as a blank control. Lactic acid thus obtained was called "residual lactic acid," or the true lactic acid, inasmuch as the interfering substances have been removed by periodate oxidation, lead precipitation, and copper-lime treatment. The molar concentration of acetol is obtained by substitution in the following formula.

[Total corrected lactic acid] − [residual lactic acid] = [acetol]

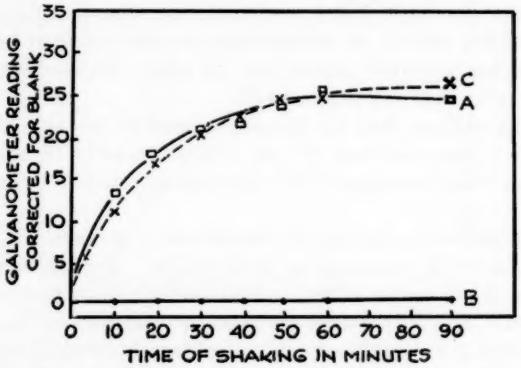

FIG. 2. The effect of the length of time of shaking on the condensation of acetol with *o*-aminobenzaldehyde. The results were obtained when acetol 6×10^{-6} M (Curve A), glucose 1.66×10^{-4} M (Curve B), and acetol 6×10^{-6} M plus glucose 1.66×10^{-4} M (Curve C) were added to the condensation mixture.

RESULTS AND DISCUSSION

Direct, Fluorometric Method

Removal of Interference Due to Glucose by Formation of Fluorescent Compound at Room Temperature—Attempts to measure the product of condensation of acetol with *o*-aminobenzaldehyde demonstrated that glucose interfered rather markedly if the reaction was carried out in a boiling water bath. This interference was probably due to the formation of acetol from glucose under these conditions (8, 9). Interference caused by glucose has been avoided by condensing acetol with *o*-aminobenzaldehyde at room temperature. Experimental evidence that validated this procedure was obtained in the following manner: Four series of tubes were prepared as described above; Series A contained acetol (8 γ), Series B glucose (3 γ),

Series C acetol (8 γ) plus glucose (3 γ), and Series D water. Three tubes of each series (A, B, C, and D) were condensed by shaking for 10 or 20 minutes, etc. for intervals of time up to 90 minutes. The data presented in Fig. 2 demonstrate that condensation of acetol with *o*-aminobenzaldehyde reached a maximum after shaking for 60 minutes (Curve A), and that glucose alone (Curve B) does not condense with *o*-aminobenzaldehyde and

TABLE I

Effect of Ethylenediaminetetraacetic Acid (EDTA) upon Removal of Interference by Copper and Manganese in Determination of Acetol

Additions	Acetol added	Galvanometer reading corrected for blank	
		Without EDTA	With 2 μmoles of EDTA
	μmole		
None	0.54	23	15
0.1 μmole copper ion	0.54	6	14
0.1 " manganese ion	0.54	9	14

TABLE II

Recovery of Acetol from Suspension of Yeast Cells, Analyzed by Fluorometric Method

Acetol added	Acetol recovered	Per cent recovery
μmoles	*μmoles*	
9.2	10.3	111
6.9	7.0	101
4.6	4.1	89
2.3	2.7	116
Average		104

furthermore does not interfere with the condensation of acetol with *o*-aminobenzaldehyde (Curve C) under these conditions.

Effect of Inorganic Ions—It is known that certain ions will influence a reaction in which *N*-methyl nicotinamide is condensed with acetone (19). Thus, it seemed desirable to test the effect of divalent and trivalent ions on this similar type of condensation reaction. 1 μmole of each of the following ions was added individually to tubes containing a constant amount of acetol and other condensation reacting agents: cobaltous, ferrous, ferric, magnesium, zinc, molybdenum, cupric, calcium, barion, vanadium, manganous, aluminum, and nickelous. Only three of these ions, namely cupric,

calcium, and manganous, were found to interfere in the determination. If these ions were added after the condensation product was formed, no inhibition was observed. Calcium, when added in a concentration of 0.1 μmole per tube, did not interfere, whereas copper and manganese in concentrations as low as 0.001 μmole per tube inhibited the complete development of fluorescence. Interference by these ions was eliminated by the addition of 2 μmoles per tube of disodium salt of ethylenediaminetetraacetic acid (Table I).

Recovery of Acetol Added to Filtrate of Bakers' Yeast—Aliquots of a standard acetol solution were added to tubes containing 1 ml. of a suspension of bakers' yeast (11 mg. per ml., dry weight of yeast). Trichloroacetic acid, 0.2 ml. of 100 per cent, was added to each tube, and the contents were mixed and then diluted to 10 ml. with water. The precipitated proteins were removed by centrifugation and acetol was determined on the resulting supernatant fluid (Table II). Under the analytical conditions employed, the method displays good reproducibility in the recovery of added amounts of acetol.

Interfering Substances—A series of compounds with biological interest was tested for possible interference in the determination of acetol by the fluorometric method. If the following compounds were added individually to the condensation reaction mixture in excess by a molar ratio of 68:1, they produced little or no interference: serine, glycerol, malic acid, ethyl alcohol, threonine, methyl glyoxal, 2,3-butylene glycol, acetone, β-hydroxybutyric acid, sodium glyoxylate, sodium glycolate, glyoxal-sodium bisulfite complex, formaldehyde, lactic acid, 1,2-propanediol, and furfural. The following substances produced slight or no interference when added individually to the reaction mixture along with a standard amount of acetol and *o*-aminobenzaldehyde: glucose 6 mg., pyridone 100 γ, *N*-methyl nicotinamide 100 γ, thymine 400 γ, nicotinic acid 100 γ, thiamine 100 γ, quinolinic acid 100 γ, and diphosphopyridine nucleotide 60 γ. The following substances interfered markedly when present in the condensation mixture in concentrations of 68:1 molar excess, but did not interfere at a concentration of 6.8:1 molar exess: acetylacetone, pyruvic acid, dihydroxyacetone, ethylacetoacetate, DL-glyceraldehyde, glycol aldehyde, and acetaldehyde. 1 γ of anthranilic acid or hydroxytryptophan added to the condensation tube produced a strong fluorescence even without addition of *o*-aminobenzaldehyde.

Diacetyl and acetylmethylcarbinol were equivalent to acetol on a molar basis, as determined in the method described. The structural similarity of the enol forms, favored by the strongly alkaline solution, of these two compounds to that of acetol is apparent. Thus, on the basis of this similarity, it might be anticipated that these substances would condense with

o-aminobenzaldehyde, inasmuch as all three compounds have a carbonyl group adjacent to a carbon atom that has an "active" hydrogen, under alkaline conditions used in this condensation method. In a preliminary experiment, it was found that one could destroy the fluorescent product of acetol by adjusting the pH to 2.6 instead of pH 8.0, whereas diacetyl and acetylmethylcarbinol condensation products showed remarkable stability at this pH.

TABLE III

Conversion of Acetol to Lactic Acid by Copper-Lime Treatment (Barker and Summerson (10))

Sample	No. of experiments	Added	Found without copper-lime	Found with copper-lime
		μmole	*μmole*	*μmole*
Lactic acid	3	0.29	0.29	0.29
Acetol	3	0.31	0	0.30

TABLE IV

Comparison of Direct and Indirect Methods for Determination of Acetol When 1,2-Propanediol and Acetol Were Added to Yeast Cells for Substrate

Substrate	μmoles acetol found*	
	Direct method	Indirect method
Acetol	95	111
"	90	102
"	83	93
"	126	136
1,2-Propanediol	11	14
"	11	13

* Micromoles per 100 mg. of dry weight of yeast cells.

Indirect, Colorimetric Method

Conversion of Acetol to Lactic Acid—Three series of test-tubes were prepared. To Series A, 1 ml. of water was added; to Series B, 1 ml. of lactic acid (0.29 μmole per ml.); and to Series C, 1 ml. of acetol (0.31 μmole per ml.). 1 ml. of 20 per cent copper sulfate solution, 8 ml. of water, and 1 gm. of calcium hydroxide were added to each tube. The contents of all the tubes were mixed and allowed to stand for 30 minutes, after which time the mixtures were centrifuged and the supernatant fluid was analyzed for lactic acid (10) along with their controls; *i.e.*, mixtures in which the copper-lime reagents were replaced by water. The results of this experi-

ment clearly show that copper-lime treatment converts acetol to lactic acid or its equivalent (Table III). In contrast to the direct method, the indirect procedure distinguishes between acetol and acetylmethylcarbinol, inasmuch as Huntress and Mulliken (14) report that acetylmethylcarbinol is converted to acetic acid by Fehling's solution. The indirect method as described is one that is perhaps more suited for use in the determination of acetol at substrate levels.

Comparison of Direct and Indirect Methods—By utilizing the two methods for the determination of acetol, the results reported in Table IV were obtained from a study (16) of the metabolism of bakers' yeast with acetol and 1,2-propanediol as the substrates. The indirect method always gives somewhat higher values, an average of about 15 per cent.

SUMMARY

Two methods for the quantitative determination of acetol are reported. A direct method, based on the condensation of acetol with *o*-aminobenzaldehyde in an alkaline solution at room temperature and the subsequent quantitative estimation of this product by fluorophotometry, is described. The second method, indirect, is based on the principle that acetol is converted to lactic acid by copper-lime treatment. Acetol is determined by difference after acetol is destroyed by periodate oxidation prior to copper-lime treatment.

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STUDIES ON THE METABOLISM OF 1,2-PROPANEDIOL PHOSPHATE IN YEAST*

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It has been reported that 1,2-propanediol phosphate[1] can be oxidized by a crystalline preparation of myogen A, a preparation which is known to contain aldolase and α-glycerophosphate dehydrogenase and perhaps other enzymes (1). The end-product of this oxidation was shown to be further oxidized by an extract of heart muscle or by crystalline D-glyceraldehyde phosphate dehydrogenase. On the basis of these observations, a series of reactions was proposed in which PDP was converted to lactic acid with acetol phosphate as a proposed intermediate. Groth and LePage (2) have found that homogenates of normal and neoplastic rat tissues under anaerobic conditions and in the presence of fluoride were able to metabolize pyruvate to yield compounds other than lactic acid. One of these compounds was demonstrated to be PDP, although other phosphate esters of unknown structure were also isolated from the reaction mixtures by paper chromatography. One of the unknown esters was suspected of being acetol phosphate. Rudney (3), using carbonyl-C^{14}-labeled acetone, demonstrated that acetone was converted to PDP and 1,2-propanediol, and to lactic acid, all of which were labeled solely in the C-2 position. It was also shown that 1-C^{14}-PD gave rise to carboxyl-C^{14}-lactic acid, indicating that conversion occurred without cleavage of the carbon chain.

The metabolism of PDP has been studied further with PDP, PD, acetol, and lactic acid as substrates for resting yeast cells. It has been found that, under appropriate conditions, any one of these metabolites can be metabolized to one or more of the others. The data obtained support the proposed reaction sequence for the metabolism of PDP, as was previously suggested (1, 4).

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[1] The following abbreviations are used: PD, 1,2-propanediol; PDP, 1,2-propanediol phosphate; TCA, trichloroacetic acid.

EXPERIMENTAL

Solutions and Substrates—Bakers' yeast was grown in a medium containing glucose, salts, and yeast extract, while being vigorously aerated. Cells were harvested by centrifugation and washed five times with saline (0.9 per cent) and then resuspended in saline so that the suspension contained approximately 14 mg. of dry yeast cells per ml. of suspension. Solutions which contained the substrates were diluted from standard stock solutions with phosphate buffer, 0.1 M, pH 7.0, and were standardized by methods previously described (5, 6).

Analytical Procedures—Lactic acid was determined by the method of Barker and Summerson (7), pyruvic acid by the method of Friedemann and Haugen (8), and formaldehyde by the chromotropic acid method as modified by Frisell *et al.* (9). PD, PDP, and acetol were determined by methods devised in this laboratory (5, 6).

Yeast cells were incubated at 37° in a standard Warburg apparatus. The main chamber of the flasks contained 1 ml. of phosphate buffer (0.1 M, pH 7.0), 0.3 ml. of substrate, 0.7 ml. of water, and the center well 0.2 ml. of KOH (10 per cent); 1 ml. of yeast suspension was tipped in from the side arm after allowing time for temperature equilibration. When sodium fluoride was used in the experiment, a solution of appropriate concentration replaced an equal volume of water. Air or 100 per cent nitrogen was used as the gas phase, depending upon whether aerobic or anaerobic conditions were desired. Enzyme action was stopped by the addition of trichloroacetic acid, 0.2 ml. of 100 per cent. Analyses for PD, PDP, acetol, and lactic acid were made on the protein-free filtrates. The data are expressed as micromoles of metabolite per 100 mg. of yeast cells, dry weight. The experiments were 4 hours in length and each value in Tables I to III is the average of four experiments.

Results

Lactic Acid As Substrate—Yeast cells were incubated anaerobically with lactic acid as the added substrate in both the absence and the presence of sodium fluoride, the concentration varying from 5×10^{-2} to 2×10^{-1} M (Table I). Lactic acid and other metabolites were quantitatively determined after an incubation period of 4 hours. Statistically, significant amounts of 1,2-propanediol (2.1 and 2.8 μmoles) were found in the presence of 10^{-1} and 2×10^{-1} M sodium fluoride. Fluoride does not cause a significant accumulation of PDP at any of the concentrations used. It is of interest that lactic acid utilization was not inhibited by fluoride. It appears likely that metabolism of lactic acid through the glycolytic reaction scheme should have been inhibited, and, by virtue of this inhibition, a greater proportion of the lactic acid utilized was recovered as PD and ace-

tol in the presence of fluoride than was recovered in similar experiments in which no fluoride had been added. These data imply that more lactic acid was utilized through the system under investigation.

Acetol As Substrate—In preliminary experiments acetol was incubated with a suspension of cells under aerobic conditions for 1.5 hours and evidence was obtained which demonstrated the reduction of acetol to PD, thus confirming the work of Farber and Nord (10). It was found, as one might have anticipated, that anaerobic conditions favor the reduction of acetol to

TABLE I

Effect of Sodium Fluoride on Formation of Metabolites from DL-*Lactic Acid by Yeast under Anaerobic Conditions*

Additions to flask	Metabolites found, μmoles			Substrate used, μmoles	Per cent total accounted for as metabolites
	PDP	PD	Acetol		
No added substrate....	0.1 (±0.1)*	1.3 (±0.8)	1.6 (±0.4)		
DL-Lactate† (45.1 μmoles).....	0.2 (±0.1)	1.3 (±0.5)	0.7 (±0.7)	65.7	3
DL-Lactate† (45.1 μmoles) + fluoride (5×10^{-2} M).....	0.7 (±0.5)	2.5 (±2.7)	26.6 (±8.2)	64.9	46
DL-Lactate† (45.1 μmoles) + fluoride (10^{-1} M).....	0.5 (±0.4)	2.1 (±0.9)	22.5 (±15.8)	72.0	35
DL-Lactate† (45.1 μmoles) + fluoride (2×10^{-1} M).....	0	2.8 (±0.4)	25.5 (±15.9)	57.1	50

* Standard error of the mean.

† The values shown have been corrected by the figures in line 1 for the contribution of yeast cells in control experiments in which no substrate was added.

PD and other metabolites (Table II). A significant amount of PDP (2.9 ± 1.3 μmoles) was also found when acetol was the added substrate under these conditions. When incubations were carried out in the presence of sodium fluoride, concentrations 5×10^{-2} and 10^{-1} M, yeast cells converted more acetol to PD.

PD and PDP As Substrates—*dl*-1,2-Propanediol and its phosphate ester were incubated with a suspension of yeast cells under aerobic conditions (Table III). A significant accumulation of acetol was found when PD was added as substrate. Previously, Goepfert (11) observed that the mold *Fusarium lini* Bolley would convert PD to acetol, Butlin and Wince (12) found that *Bacterium suboxydans* would perform this oxidation and Levine and Krampitz (13) made similar observations with a soil diph-

theroid. A significant amount of lactic acid was also found, which suggests that PD is oxidized to lactic acid, while there was only suggestive evidence that PD was phosphorylated under these conditions. The evi-

TABLE II

Effect of Sodium Fluoride on Metabolites Formed from Acetol by Yeast under Anaerobic Conditions

Additions to flask	Metabolites found, μmoles			Substrate used, μmoles	Per cent total accounted for as metabolites
	PDP	PD	Lactic acid		
No added substrate.....	0.4 (±0.2)*	0.9 (±0.3)	2.7 (±1.3)		
Acetol† (18.4 μmoles)...	2.9 (±1.3)	32.3 (±7.7)	4.6 (±1.5)	54.2	73
" † (18.4 μmoles) + fluoride (5×10^{-2} M)..	4.6 (±1.9)	48.2 (±9.5)	2.5 (±1.1)	67.0	82
Acetol† (18.4 μmoles) + fluoride (10^{-1} M).....	4.1 (±2.9)	52.9 (±4.7)	4.0 (±0.9)	74.2	82
Acetol† (18.4 μmoles) + fluoride (2×10^{-1} M)..	0.4 (±0.4)	32.8 (±1.9)	4.0 (±2.5)	36.7	101

* Standard error of the mean.

† The values shown have been corrected by the figures in line 1 for the contribution made by yeast cells in control experiments in which no substrate was added.

TABLE III

Effect of Sodium Fluoride on Metabolites Formed from PD and PDP by Yeast under Aerobic Conditions

Additions to flask	Metabolites found, μmoles				Added substrate used, μmoles	Per cent total accounted for as metabolites
	PDP	PD	Acetol	Lactic acid		
No added substrate.....	1.0 (±0.4)*	1.7 (±0.4)	0.6 (±0.2)	0.6 (±0.2)	0	0
PDP† (15.7 μmoles).....		3.3 (±1.9)	0	17.1 (±4.9)	62.6	33
PDP† (15.7 μmoles) + fluoride (10^{-1} M)†..		5.6 (±2.3)	0	10.5 (±1.9)	31.7	51
PD† (17.1 μmoles).....	3.5 (±2.2)		13.6 (±1.0)	5.1 (±0.6)	36.9	60
PD† (17.1 μmoles) + fluoride (10^{-1} M)..	5.8 (±3.6)		8.9 (±1.5)	2.1 (±1.5)	35.7	47

* Standard error of the mean.

† The values shown have been corrected by the figures in line 1 for the contribution made by yeast cells in control experiments in which no substrate was added.

dence suggests that PDP may be hydrolyzed by phosphatase to PD during the incubation with yeast cells, although the data are inconclusive. It was not possible, with the data obtained, to determine whether acetol was formed by dehydrogenation of PDP and subsequent dephosphorylation of acetol phosphate or dehydrogenation of PD, which is formed by phosphatase action on PDP, although lactic acid was formed from PDP.

Both PD and PDP were incubated with yeast cells, under aerobic conditions, in the presence of sodium fluoride, 10^{-1} M (Table III). There was a significant amount of PD and lactic acid formed from PDP; however, no acetol accumulated. Although there was lactic acid formed from PD in the absence of fluoride, this inhibitor seemed to prevent the accumulation of lactic acid. Fluoride did not prevent the conversion of PD to acetol. This observation is consistent with the data obtained from experiments in which lactic acid was the substrate.

Analyses for Other Metabolites—The protein-free filtrates were also analyzed for the appearance of keto acids by the direct method of Friedemann and Haugen (8). No significant accumulation of keto acids was observed after corrections were made for contributions made by acetol (14). Protein-free filtrates were analyzed to determine the concentration of compounds that yield formaldehyde upon oxidation with periodate and, subsequently, formaldehyde was quantitatively determined (7). It was found in many instances that more "formaldehyde-yielding" compounds appeared than could be accounted for on the basis of the contribution made by propanediol, acetol, and endogenous substrates (14).

DISCUSSION

It has been previously expressed that PDP is metabolized to lactic acid by a pathway that involves acetol phosphate (Miller, Huggins, and Arai (1)). The interconvertibility of PDP, acetol, and lactic acid is taken as evidence that acetol is an intermediate in a sequence of reactions in which PDP is converted to lactic acid.

It was demonstrated that PD and acetol accumulated when lactic acid was incubated anaerobically with yeast cells. The evidence that lactic acid is converted to PD and acetol, under anaerobic conditions, was not unequivocal, in that the effect noted might possibly have been an indirect one. Sodium fluoride, at concentrations high enough to inhibit glycolysis, did not inhibit lactic acid utilization, which is in confirmation of data previously reported by Miller and Olson (15) that demonstrated the insensitivity of cardiac muscle slices to fluoride poisoning with regard to lactic acid utilization. Although fluoride appears to favor the accumulation of acetol as well as PD, it is probable that this effect is the result of inhibition of the dissimulation of PDP.

More PD was formed from acetol under anaerobic conditions than under aerobic conditions. In this respect acetol may be similar to pyruvic acid in its ability to serve as a hydrogen acceptor. The addition of fluoride to the incubation mixture appeared to increase the accumulation of PD, which is in support of the view that fluoride blocks the further dissimulation of PDP. The observation that fluoride (10^{-1} M) failed to inhibit the total acetol utilization is in agreement with this concept. However, it is possible that fluoride prevented the formation of pyruvic acid from endogenous substrates via the glycolytic system, and thus acetol may have served only as a hydrogen acceptor in place of pyruvic acid. The accumulation of PDP as well as PD suggests that acetol may be phosphorylated prior to reduction of PDP.

Propanediol is oxidized to acetol, but less acetol accumulates in the presence of fluoride (10^{-1} M). This observation is apparently not consistent with the observed insensitivity of the acetol to fluoride inhibition. It is likely that fluoride does not directly effect either acetol or PD metabolism but rather that the effect may be on a common intermediate such as PDP. The oxidation of PD to lactic acid is in agreement with the observations of Newman *et al.* (16), who found that lactic acid accumulated in the perfused fluid when a cat liver was perfused with blood to which PD had been added. Also their findings are consistent with the observations of Rudney (3), who isolated carboxyl-labeled lactic acid from an incubation mixture in which propanediol-1-C^{14} was used as substrate.

Curiously enough, fluoride did not prevent the accumulation of significant amounts of PD or lactic acid in the incubation mixture in which PDP was the added substrate, although no acetol accumulation was demonstrable in either the presence or the absence of the inhibitor. If PDP were an obligatory intermediate for PD utilization, one would have anticipated that acetol would have accumulated when PDP was added as the substrate, just as was observed when PD was the added substrate. However, it seems unlikely that PDP was converted to lactic acid by virtue of its conversion to a glycolytic intermediate higher than pyruvic acid, since fluoride (10^{-1} M) would certainly have reduced its conversion to lactic acid by this pathway.

It appears that this investigation has given support to the original postulate (1) that PDP is metabolized to lactic acid by a sequence of reactions which includes acetol or its phosphate ester as an intermediate. The metabolism of PDP in yeast cells may be considerably more complex than was anticipated from earlier work with other systems.

SUMMARY

The postulated metabolic pathway whereby 1,2-propanediol is metabolized to lactic acid via acetol or its phosphate ester was studied by using

bakers' yeast cells. Propanediol, propanediol phosphate, acetol, and lactic acid were used as substrates for the cells, and the incubation mixtures were analyzed for metabolites of the proposed reaction sequence as well as for the remaining substrate. The data support the reaction sequence proposed for the metabolism of 1,2-propanediol phosphate (1).

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RATES OF DEOXYRIBONUCLEIC ACID FORMATION AND CELL PRODUCTION IN REGENERATING RAT LIVER

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The parallel between mitotic activity and the rate of P^{32} uptake into deoxyribonucleic acid (DNA) (1, 2) is explained by the incorporation of phosphate into the new DNA synthesized for each mitosis. The *classical* theory holds that the amount of new DNA thus formed for mitosis is just equal to that previously present in the cell. However, data obtained in this laboratory indicated that twice as much P^{32}-labeled DNA was formed as could be accounted for by the net increase in DNA content at mitosis (3, 4). This result suggested that the mitotic process involved not only formation of additional DNA but also replacement of the previously present DNA. This *renewal* theory was supported by Barnum *et al.* (5), whereas recent observations made on bacteria (6, 7) and growing liver (8) supported the classical theory.

The validity of the DNA renewal theory was checked by a critical investigation of the methods used: (1) The deoxyribonucleic acid phosphorus (DNA P) isolated by a modified (9) Schmidt and Thannhauser procedure (10) was found not to be significantly contaminated by other phosphorus compounds (9). (2) The specific activity of the acid-soluble phosphorus fraction, which in our studies was taken to be that of the DNA precursor, was compared to that of the free nucleotides present (the precursors of nucleic acids (11–14)) and found to be quite similar (15). Recalculations of previous results (3, 4) with the nucleotides instead of the whole acid-soluble fraction to represent the DNA P precursor gave negligibly higher rates of DNA P formation. Therefore, the published figures for the rates of DNA P formation, or at least their order of magnitude, are presumed to be correct. (3) The colchicine used to measure the rate of cell production (16, 17) has been shown in recent investigations to interfere with the initiation of mitosis and to induce the disintegration of some of the arrested mitoses. Thus, the estimates of cell formation obtained by use of the colchicine technique may be too low.

Accordingly, a tissue in which the rate of cell production could be measured directly was sought for study. Since active liver regeneration after partial hepatectomy leads to accumulation of a large number of new cells within a definite time period, such cell production can be measured by de-

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termining increases in weight and DNA P^{31} content of the remaining liver tissue. In the present work the rates of P^{32} uptake by DNA were estimated by the same methods as in previous investigations (3, 4) and were directly compared to the net increases in DNA P^{31} content.

Materials and Methods

Forty-eight adult male albino rats weighing 220 gm. on the average (range 190 to 230 gm.) were divided into groups of six or twelve animals. The rats were injected subcutaneously with 150 μc. of P^{32}-phosphate and partially hepatectomized 2 hours later by removal of the median and left lateral lobes of the liver (18). All hepatectomies were performed between 11 a.m. and 2 p.m. The animals were maintained on a diet of Purina fox chow. A 5 per cent glucose solution was given as drinking water after hepatectomy.

At various intervals after the operation, the animals (twelve rats at 0 hour, six at 12 and 18 hours, twelve at 24 hours, and six at 36 and 48 hours) were anesthetized with ether and exsanguinated via abdominal aortic puncture. The remaining portions of liver were excised and weighed after placing a small aliquot in Susa fluid for histological study. The livers of groups of six rats were pooled and kept at $-10°$ until the last group of animals had been sacrificed. After thawing and mincing in a Plexiglass squeezer, 3 gm. of liver pulp were weighed out of each pool and homogenized with 40 ml. of ice-cold 10 per cent trichloroacetic acid. The supernatant fluid (acid-soluble fraction) was obtained by filtration; half was used to investigate the whole acid-soluble phosphorus fraction, the other half for the separation of inorganic from organic phosphorus (19). The DNA was isolated chemically from the residue (acid-insoluble fraction) by the modified (9) Schmidt and Thannhauser procedure (10). This procedure, which entailed a regular loss of 20 to 25 per cent of the DNA (unpublished data), was sufficiently consistent for the values obtained to be considered valid, relative to one another.

The total phosphorus content (P^{31}) of the various fractions was determined (20), following digestion with concentrated sulfuric acid, except in the case of the inorganic fraction, which was estimated directly. Radiophosphorus content was determined in triplicate 0.5 ml. aliquots of the digested solutions. The injected dose of P^{32} was calculated from aliquots of the P^{32} solution used for the injections. The specific activities of the phosphorus fractions were computed as percentages of the injected dose per microgram of phosphorus, the figure for the injected dose being adjusted for 200 gm. of body weight as in the previous work (3, 4).

To calculate the rates of DNAP formation, simple calculations were used. More refined mathematical analysis by one of us (N. J. N.) led to the same conclusions as outlined below.

RESULTS AND DISCUSSION

In the zero hour group, it was found that the median and left lateral liver lobes accounted for 69.1 per cent and the remaining lobes for 30.9 per cent of the mean liver weight (8.85 gm.). Postoperatively, the weight of the remaining lobes increased progressively, starting 24 hours after operation (Table I). Microscopic examination revealed that mitoses were few at 0, 12, and 18 hours, but were numerous at 24 hours and thereafter. These observations were in agreement with previous reports (18, 21–26).

TABLE I

Liver Weights, Amount of DNA P, and Specific Activities of Phosphorus Fractions at Different Time Intervals Following Partial Hepatectomy

Time after partial hepatectomy	Weight of liver tissue present*	Amount of DNA P†		Specific activities of phosphorus fractions‡§			
		Fresh liver	Total liver tissue	Acid-soluble P			DNA P
				Whole fraction	Inorganic P	Organic P	
hrs.	*gm.*	*mg. per gm.*	*mg.*				
0	2.74	0.149	0.408		13.28	10.46	0.036
12	2.60	0.162	0.421	3.74	4.23	3.62	0.044
18	2.84	0.156	0.443	2.80	3.33	2.66	0.050
24	3.41	0.153	0.522	3.28	2.82	2.18	0.247
36	4.12	0.158	0.651	2.53	1.91	2.13	0.743
48	4.81	0.158	0.760	2.04	1.71	2.03	0.908

* The value at zero time represents the total weight of the small lobes left immediately after partial hepatectomy.

† The figures in each column must be considered relative to one another and not as absolute values (see the text).

‡ The radiophosphorus was injected 2 hours before partial hepatectomy.

§ Specific activity values are expressed as percentages of the injected dose of P^{32} per microgram of $P^{31} \times 10^{-4}$, the figure for the injected dose being adjusted for 200 gm. of body weight.

The uncorrected values for the DNA P (P^{31}) content of regenerating liver (Table I), like the accurate DNA values reported for the 24, 36, and 48 hour intervals by Ultmann *et al.* (26), showed that the amount of DNA P per gm. of fresh liver remained practically constant at the different periods studied and, as a consequence, the DNA P content increased in proportion to the liver weight (Table I).

The specific activities of the whole acid-soluble phosphorus fraction as well as those of its inorganic and organic components (Table I) were similar to the specific activity values obtained for livers of intact rats at corresponding time intervals after P^{32} injection (3); therefore, hepatectomy had not significantly altered the rate of phosphorus deposition in these fractions.

At the end of 12 hours, the specific activities of the various acid-soluble fractions had reached the same order of magnitude, and later each showed similar slow and progressive decreases with time (Table I).

The specific activity of DNA P remained low until 18 hours after hepatectomy, but during the 18 to 24 hour period and thereafter rose steadily (Table I). Thus, active DNA synthesis started about 18 hours after partial hepatectomy (27, 28).

To compare the increase in liver weight (or liver DNA P) with the increase in newly formed DNA P, it was necessary to consider only the data obtained after significant cell formation began; that is, after the 18th hour

TABLE II

Increases in Weight and Total DNA P Content of Rat Liver As Compared to Amounts of Labeled DNA P Formed during Various Time Intervals Following Partial Hepatectomy

The results are expressed as per cent of the DNA P present at the end of each time period.

Time interval	Increase in liver weight	Increase in amount of DNA P per liver	Increase in amount of newly formed DNA P $(n \times 100)/(m + s)$
hrs.			
18–24	16.7	15.1	8.3
18–36	31.1	32.0	31.5
18–48	41.0	41.7	40.3
24–36	17.2	19.8	25.9
24–48	29.1	31.3	35.3
36–48	14.3	14.3	12.3

following operation. All possible experimental periods between 18 and 48 hours were, therefore, examined in the calculations (Table II, first column). For any given period, the increases in liver weight were conveniently expressed as percentages of the total amount present at the *end* of the period (second column). Increases in the amount of DNA P per liver were expressed in the same manner (third column). Here again it is apparent that liver weight and DNA P increase in parallel fashion.

The purpose of the present work is to compare the rate of addition of DNA P as presented in the third column of Table II to the rate of uptake of P^{32} by DNA. To calculate the latter, let us consider that the DNA P found at the end of each time period (with its specific activity designated as "γ") is composed of the DNA P present at the beginning of the period (in an amount m and with a specific activity α) and the DNA P formed

during the period (in an amount n and with the specific activity of the precursor, that is the mean specific activity, β, of the organic acid-soluble phosphorus during that period). This can be expressed by the general equation for mixtures, $m\alpha + n\beta = (m + n)\gamma$.

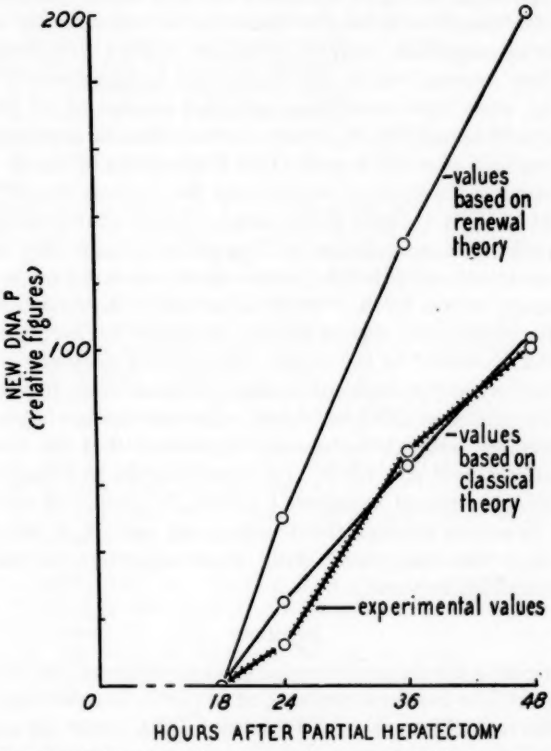

FIG. 1. Experimental values for the newly formed DNA as compared to the values calculated on the basis of the classical and renewal theories. The experimental data support the classical theory.

The problem is to estimate how much of the DNA P present at the end of a period is newly formed; that is, to calculate the ratio of new to total DNA P, $n/(m + n)$, which may be obtained from the above equation, in which α, β, and γ are known, by adding $-n\alpha$ to both sides. The resulting equation is $n/(m + n) = (\gamma - \alpha)/(\beta - \alpha)$. The calculation of this ratio for each period is tabulated as the "Increase in amount of newly formed DNA P" (Table II, fourth column).

The rates of appearance of new DNA P thus calculated should be either equal to or double the rate of addition of DNA P (Table II, third column) according to whether the classical or the renewal theory of P^{32} incorporation into DNA is correct. Examination of Table II revealed that in no case were the figures in the fourth column of Table II double those in the third column. On the other hand, the figures in the two columns were of the same order of magnitude, with the exception of the result obtained at the 18 to 24 hour interval, which may be ascribed to experimental variations.

Similarly, when these results are expressed as amounts of *new* DNA P formed after 18 hours (Fig. 1), it may be seen that the experimentally determined amount of newly formed DNA P appearing in regenerating liver is about equal to the amount predicted by the classical theory; that is, to the amount of DNA P added to the tissue. These results confirmed those obtained under similar conditions by Nygaard and Rusch (29), whose work came to our attention while the present report was being prepared.

The amount of new DNA P which is formed in the liver during active regenerative growth may thus be entirely accounted for by the net increase in the DNA P content of the organ. Since DNA synthesis is known to occur exclusively at the duplication stage of mitosis (30), this would mean that only the additional DNA which is then formed becomes labeled. These results, therefore, support the classical hypothesis that the dividing cells contain half old, half new DNA, and contradict the hypothesis that preexisting DNA is replaced at mitosis by twice its amount of new DNA, as suggested by results obtained previously in our own (3, 4) and other laboratories (5). Thus the present work offers support to the concept that DNA is metabolically stable.

SUMMARY

The increase in the deoxyribonucleic acid phosphorus content of the regenerating rat liver has been measured after partial hepatectomy and compared to the rate of formation of P^{32}-labeled DNA under the same conditions.

The results showed that the total amount of labeled DNA P which appears in the liver during regenerative growth may be accounted for by the net increase in the DNA P content of the organ.

The present data support the view that the duplication of DNA at mitosis is not due to a replacement of the preexisting DNA by twice its amount of new DNA, but rather to the addition of an amount of new DNA equal to that already present.

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A SENSITIVE, ACCURATE SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF MAGNESIUM IN SERUM*

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Numerous chemical procedures have been reported for the determination of magnesium. Precipitation methods (1, 2), indirect titration methods (3), and colorimetric methods such as the titan yellow procedure which depends upon lake formation (4, 5) have not proved entirely satisfactory for biological work, owing to a lack of either sensitivity, reproducibility, or accuracy. The majority of the flame photometric methods for measuring magnesium, for example the procedure of Kapuscinski *et al.* (6), suffer from a lack of sensitivity and resultant high background. In a recent paper, Davis (7) has circumvented these difficulties, but only by means of special equipment, *vis.* photomultiplier and oxyhydrogen flame accessories.

With the growing emphasis in our laboratory and elsewhere on cation distribution in normal and pathological states, a need for a simple, accurate, and sensitive method for determining magnesium in biological material became apparent. We were particularly interested in the procedures for determining magnesium in water by using Eriochrome Black T (8, 9), since they involve a true solution of a colored magnesium complex in which the magnesium concentration is directly proportional to the color intensity.

The method reported here is, in principle, similar to that of Young *et al.* (9), modified for use with biological material. It is both sensitive and accurate and has the additional advantage that a calcium determination can be carried out on the same sample of serum as that used for the determination of magnesium.

EXPERIMENTAL

Reagents—

1. Calcium precipitating reagent. To 1 volume of saturated ammonium oxalate, 1 volume of 1 M sodium acetate buffer, pH 5.0, and 3 volumes of distilled water are added.

2. Trichloroacetic acid. 62.5 gm. of trichloroacetic acid are made up to 1 liter with water.

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3. Piperidine buffer, pH 12.0 to 12.2. Piperidine, 15.6 ml., is diluted with about 70 ml. of water. After mixing, 1.5 ml. of concentrated HCl are added and the solution diluted to about 100 ml. with water. If necessary, the buffer may be adjusted with HCl or piperidine so that the pH is between 12.0 and 12.2. A Beckman model G pH meter, equipped with a saturated calomel electrode and a Beckman type E glass electrode, was used and standardized with 0.04 M borax at pH 9.18. The age of the piperidine buffer should not exceed 48 hours.

4. Magnesium standard. For the stock solution, powdered metallic Mg, 100 mg., is placed in a 100 ml. flask and dissolved in 5 ml. of water and 2.5 ml. of concentrated HCl. This solution is diluted to 100 ml. with water and 2 ml. of chloroform are added as a preservative. Working standards are prepared from the stock solution each day.

5. Ethanol. Commercial 95 per cent ethanol, transported in metal containers, was found to be unsuitable for the procedure. Therefore, 95 per cent ethanol is distilled in an all-glass apparatus and then stored in glass bottles.

6. Dye solution. Eriochrome Black T (Hach Chemical Company, Ames, Iowa), 10 mg., is transferred to a 25 ml. volumetric flask, 1.5 ml. of water being used. Piperidine buffer, 0.1 ml., is added and the solution brought to the 25 ml. mark with redistilled 95 per cent ethanol. After a brief shaking, the dye reagent is transferred to a 100 ml. brown glass bottle fitted with a vented stopper encased in Parafilm. The solution is shaken mechanically for 30 minutes and then stored in the dark until ready for use.

Analytical Procedure

Precipitation of Ca—To 1 ml. of freshly drawn serum or plasma (heparin is used as an anticoagulant) in a 5 ml. conical centrifuge tube, 2 ml. of Ca precipitating reagent are added, and mixing is obtained by gently swirling the tube. The centrifuge tube is tightly covered with Parafilm and stored overnight at 4°. The following morning the precipitate is dislodged from the walls by vigorously rolling the tube between the palms of the hands. The tube is then centrifuged[1] in the cold for 20 minutes at 1200 × *g*, and from the tube, which must remain cold, 1 ml. of supernatant fluid is carefully pipetted out and placed in a 15 ml. centrifuge tube for Mg determination. If a Ca determination is to be made, the 5 ml. centrifuge tube is recentrifuged at 1200 × *g* before proceeding further.

Determination of Mg—To 1 ml. of the supernatant fluid from the precipitation of Ca, 4 ml. of 6.25 per cent trichloroacetic acid are added and mixed by gentle swirling. The protein precipitate is allowed to form for at least

[1] Special carriers were obtained from the International Centrifuge Company, Boston, Massachusetts.

5 minutes and the tube is then centrifuged for 15 minutes at 1400 × *g*. A 2 ml. aliquot of the supernatant fluid is pipetted into a 10 ml. volumetric flask. 3 ml. of piperidine buffer, 3 ml. of 95 per cent ethanol, and 1 ml. of dye solution are added to the flask and the contents brought to volume with water. The flask is vigorously shaken and then stored in the dark for 30 minutes. A 1 ml. water blank and 1 ml. dilute Mg standards in water (10 to 40 γ to correspond with 1 ml. of serum) are simultaneously car-

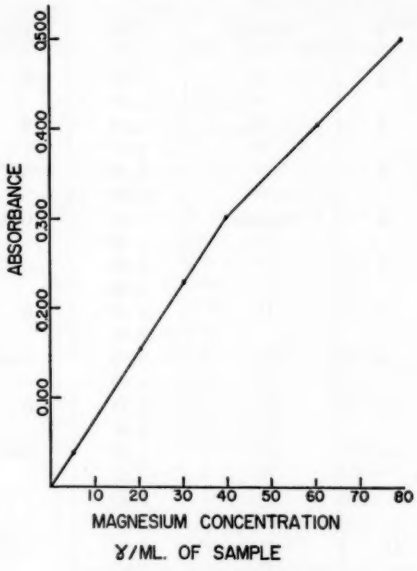

FIG. 1. Concentration curve

ried through the entire procedure, beginning with the addition of the buffered oxalate reagent.

The unknown and the standards are read against the reagent blank in a Beckman model DU spectrophotometer in Corex 1 cm. cells at 520 mμ. The sensitivity knob is kept about one-half turn from its fully counterclockwise position in order to obtain a narrow slit width (about 0.4 mm.).

Validity of Method

Sensitivity of Range—The sensitivity of the method allows for the determination of Mg in amounts as low as 5 γ per ml. of serum (0.5 mg. per cent). As Fig. 1 reveals, the absorption curve is linear up to a concentration of 40 γ per ml. The absorbance is also directly proportional to the

quantity of Mg from 40 to 80 γ per ml., but there is a break in the curve at 40 γ.

TABLE I

Reproducibility of Magnesium Determinations in Replicate Dog and Rabbit Plasma Samples

Species	Sample No.	Source of blood	Magnesium, mg. per cent	Average difference	Per cent difference
Dog	1	Arterial	2.40	0.015	0.63
		"	2.37		
	2	"	2.40	0	0
		"	2.40		
	3	"	2.25	0	0
		"	2.25		
	4	"	2.60	0	0
		"	2.60		
	5	Venous	2.15	0.035	1.65
		"	2.08		
	6	"	2.20	0.1	4.3
		"	2.40		
	7	"	2.30	0	0
		"	2.30		
	8	"	2.40	0.05	2.04
		"	2.50		
	9	"	2.45	0	0
		"	2.45		
	10	"	2.20	0.03	1.3
		"	2.23		
		"	2.27		
		"	2.27		
	11	"	2.60	0.025	0.95
		"	2.65		
	12	"	2.30		
		"	2.35	0.03	1.0
		"	2.35		
Rabbit	1	"	3.25	0	0
		"	3.25		
	2	"	2.72	0.06	2.2
		"	2.60		
	3	"	3.02	0.015	0.5
		"	3.05		
	4	"	3.00	0.05	1.6
		"	3.10		

Stability of Color—The color development is complete within 30 minutes after the addition of the Eriochrome Black T reagent, and the measured absorbances remain constant up to 60 minutes. One factor in the stability

of the final color is the age of the dye solution. The dye reagent attains a stable value 30 minutes after preparation and remains stable for an additional 90 minutes.

Effect of Temperature—Three sets of standards were prepared and the color allowed to develop at 20.5°, 28°, and 37.5°. There was no difference in readings between the three sets of standards, indicating that temperature, within the range of 20.5–37.5°, has no effect upon color development.

Effect of pH—The degree of color development is a function of the pH of the final solution (8, 10). The pH of the piperidine buffer must, therefore, be rather rigorously controlled. There is no difference in the absorbancy

TABLE II

Recovery of Magnesium

Serum sample	Magnesium added	Magnesium found	Magnesium calculated	Per cent difference
	γ per ml.	*mg. per cent*	*mg. per cent*	
A	0	2.50		
"	5	2.95	3.00	−1.7
"	10	3.50	3.50	0
"	15	3.98	4.00	−0.5
B	0	2.09		
"	5	2.63	2.59	+1.5
"	10	3.00	3.09	−2.9
"	15	3.55	3.59	−1.1
C	0	2.33		
"	5	2.80	2.83	−1.1
"	10	3.35	3.33	+0.6
"	15	3.93	3.83	+2.6
D	0	2.23		
"	5	2.80	2.73	+2.5

measurements with piperidine buffers ranging between pH 12.0 and 12.2. According to the procedure outlined above, the final pH is between 11.4 and 11.5 and the absorbancy measurements are constant within this range. Since the final pH of the reaction is determined not only by the piperidine buffer but also by the amount of ethanol added, this latter addition must be made accurately.

Effect of Oxalate—A comparison of standards prepared with and without oxalate showed that oxalate decreased the measured absorbance; the greater the oxalate concentration, the lower the absorbancy values.

Effect of Other Ions—Eriochrome Black T forms complexes with ions other than Mg, notably Ca. Although the latter could produce a considerable error in the determination of Mg in serum, the method of precipitating Ca used here has given reproducible results not only in replicate de-

terminations of Mg (Table I) but also in subsequent determinations of Ca by flame photometry (11). Quantitative recovery of known amounts of Mg (Table II) and of Ca added to serum was also indicative of complete Ca precipitation.

Addition of K and Na as chlorides in amounts equivalent to and above the normal range found in serum had no effect on the color development. Addition of Fe as $Fe_2(SO_4)_3$ and $FeSO_4$ in amounts corresponding to 10 times the upper limit of Fe in plasma (10×0.2 mg. per cent) had no effect on the measured absorbance of Mg or the reagent blank.

Reproducibility and Recovery—In Table I is a series of replicate determinations of Mg in dog and rabbit plasma. The greatest error found among the replicates was 4.3 per cent, but in only six out of sixteen determinations was the error greater than 1 per cent.

In order to test the accuracy of the method in recovering Mg from plasma, a series of experiments was run in which known amounts of Mg were added to aliquots of the same sample of plasma before the precipitation of Ca. The results of four of these experiments are given in Table II. It may be noted that the error in recovery never exceeded ± 3 per cent.

DISCUSSION

The presence of oxalate in the reaction mixture affects the measured absorbance. However, if the standard and the reagent blank are treated in exactly the same manner as the sera, this effect of oxalate is canceled. If the quantity of the aliquot taken from the supernatant fluid of either the Ca or the protein precipitation is increased, the amount of oxalate present in the final mixture is automatically increased. Therefore, any increase in sensitivity that might be hoped for is largely canceled by the interference of the increased oxalate concentration. Also, when the amount of the aliquots taken for analysis is increased, the absorption curve is linear over a shorter range. Satisfactory linearity and reproducibility are obtained only with the final oxalate concentration produced by the procedure outlined above.

The major factor in the accuracy and reproducibility of the Mg determination is the completeness of the Ca precipitation. Various other methods of precipitating Ca were tried (8, 12), but the results proved unsatisfactory. Even with the method outlined above, extreme care must be taken to make certain that the Ca oxalate is well sedimented before aliquots of the supernatant fluid are removed for Mg determination. The samples should be recentrifuged if necessary. In addition, unsatisfactory replication of the results was obtained when aliquots for Mg determination were taken from supernatant fluids which had been allowed to warm up to room temperature. Therefore, sampling for the determination of Mg was carried out

either in the cold room or at room temperature by keeping the centrifuge tubes immersed in ice.

Although we found that Na, K, and Fe in concentrations well above those found in normal plasma did not interfere with the measurement of Mg, Young *et al.* (9) reported slight interference of Na and K in their method for the simultaneous determination of Ca and Mg with Eriochrome Black T. The error produced by Na and K was greater for Ca than for Mg and may have been due to the fact that their determinations were based on absorbancy measurements made at two pH values, 9.52 and 11.70. These same authors found considerable error in their measurement of Mg in the presence of Fe. In their procedure, Young and his coworkers add the dye reagent to the reaction mixture before the addition of the buffer. Diehl *et al.* (13) have shown that Fe will combine with Eriochrome Black T when the indicator is added before the buffer. However, when the buffer is added before the dye, as in our procedure, the Fe is precipitated as the hydroxide and is no longer a source of error.

Young and Sweet (10) have presented evidence which indicates that Eriochrome Black T forms 1:1, 2:1, and 3:1 complexes with Mg. They found that the formation of higher complexes increases as the pH increases, and that at pH 11.7 the 3:1 complex exists. The absorbance at 520 mμ is consequently greatest for this high pH. In preliminary investigations, by the method of Harvey *et al.* (8), we used a buffer at pH 10.1 and found that the absorption curve was linear for only a very narrow range of Mg concentrations. We also noted that increasing the amount of dye reagent increased the absorbancy measurements; *i.e.*, increased the slope of the curve. However, the amount of dye reagent which can be used is self-limiting, owing to the color of the Eriochrome Black T, and therefore linearity could not be attained simply by increasing the amount of dye reagent further. These preliminary investigations indicated that at pH 10.1 no one complex of Mg and dye predominated, and that a change in the molar ratio of Mg to dye reagent resulted in a change in the ratio of the mixture of the complexes present. We, therefore, preferred to use the higher pH value of 11.5, at which value only the 3:1 complex apparently exists (10). However, the break in the absorption curve (Fig. 1) at 40 γ per ml. may be indicative of the fact that above this concentration of Mg a mixture of complexes is again encountered.

The absorbancy values of the Mg standards are usually reproducible within 2 per cent from day to day. Important factors which may affect the standard reading are the age of the stock piperidine and the actual preparation of the reagents. In order to obtain results that are always comparable, we suggest that at least two, preferably three, known concentrations of Mg be run with all unknowns. This practice will correct for any

error that may arise from an inadvertent change in the pH of the buffer or in the concentration of the dye.

SUMMARY

A spectrophotometric method is presented for the determination of magnesium in serum, in amounts as low as 5 γ per ml., with Eriochrome Black T as the dye reagent. This method allows for the determination of calcium as well as magnesium on a 1 ml. sample of serum or plasma. In the sixteen values for normal dog and rabbit sera reported, the average error among magnesium replicate determinations was 0.95 per cent. The average error for the recovery of known amounts of magnesium from serum was 1.45 per cent.

Addendum—Since this article was prepared for publication, a report of another method for the determination of Mg by using Eriochrome Black T (14) has come to our attention. In this method a buffer is used at pH 10.1 and requires 2 ml. of serum for the determination. In the procedure filter paper is utilized, a practice which, in our experience, increased the Mg content of the sample. The accuracy of Smith's method is ±5 per cent.

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PYRIMIDINE BIOSYNTHESIS IN ESCHERICHIA COLI*

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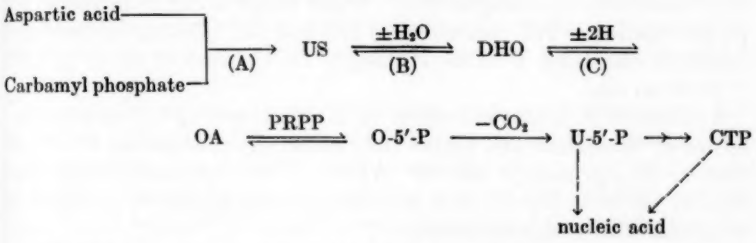
The recent great interest in the formation and function of nucleic acids has caused a similar interest in the synthesis of such component parts of nucleic acid as the pyrimidines. Implicated in pyrimidine biosynthesis have been the compounds aspartic acid (1, 2), ureidosuccinic acid (US) (1–5), dihydroorotic acid (DHO) (6), orotic acid (OA) (4–8), 5-phosphoribosylpyrophosphate (PRPP) (9), orotidine-5′-phosphate (O-5′-P) (10, 11), uridine-5′-phosphate (U-5′-P) (10, 11), and cytidine triphosphate (CTP) (12).

A composite scheme of pyrimidine biosynthesis drawn from the above references can be summarized in the accompanying diagram, all steps with solid arrows representing known enzymatic reactions.



The enzyme found for step (A) has been named ureidosuccinic acid synthetase by Jones *et al.* (1), and the enzymes for steps (B) and (C) are named, respectively, dihydroorotase (5) and dihydroorotic acid dehydrogenase (6) by Lieberman and Kornberg.

The study of pyrimidine biosynthesis by Lieberman, Kornberg, and Simms has been outstanding in revealing enzymatically the whole pathway from step (B) on. One limitation to the conclusions drawn from their earlier work (5, 6) concerning the steps from US through OA, however, is that organisms (*Zymobacterium oroticum* (6) and some corynebacteria (13)) grown on OA media were used as source material for enzyme studies. Un-

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† Abraham Rosenberg Research Fellow during the course of this work.

der such growth conditions enzymes able to degrade OA may be formed adaptively to provide energy and constituents for cell growth, and such enzymes may be unrelated to normal pyrimidine biosynthesis.

It was desired here to determine both qualitatively and quantitatively whether the early steps in the biosynthesis proceed as shown above or by an alternative mechanism as suggested by the work of Mitchell *et al.* with *Neurospora crassa* mutants (7). *Escherichia coli*, strain B, and two pyrimidine-requiring *E. coli* mutants were used for this study, grown under contions for which all enzymes studied would presumably be present only to synthesize pyrimidines.

Materials and Methods

Uracil, OA, and DL-aspartic acid-4-C^{14} were purchased from the California Foundation for Biochemical Research. DL-Ureidosuccinic acid prepared by the method of Nyc and Mitchell (14) had a melting point at 176.3° (decomposed). A mixed melting point with DL-US obtained from Dr. H. A. Barker showed no depression.

L-Dihydroorotic acid was prepared by the method of Miller *et al.* (15) from carbethoxy-L-asparagine (16). Twice recrystallized from water, the product melted at 173° (uncorrected) and had the following composition: $C_5H_6O_4N_2$ calculated, C 37.98, H 3.93, N 17.71; found, C 38.17, H 3.97, N 17.60 per cent.

A culture of *Z. oroticum*, isolated by Dr. A. Kornberg (17) and characterized by Wachsman and Barker (18), was kindly provided by Dr. H. A. Barker. *E. coli* mutant 550-460 (ATCC 11548) and mutant 6386 (isolated by Dr. B. D. Davis), both of which require pyrimidines, were present in the collection of this Laboratory.

C^{14} was determined in a gas flow Geiger-Müller counter with platinum plates.

Bacterial Medium and Growth Conditions—E. coli strains were grown with aeration by swirling at 37° in a minimal medium of the following composition in gm. per liter: 7 K_2HPO_4, 2 KH_2PO_4, 0.5 sodium citrate·$5H_2O$, 0.1 $MgSO_4 \cdot 7H_2O$, 1 $(NH_4)_2SO_4$, 6.3 glycerol (or 2.5 glucose) (19). In some cases the medium was supplemented with 2×10^{-4} M uracil or OA as growth factor.

Z. oroticum cultures were grown on OA medium, harvested, suspended in the presence of OA *in vacuo*, washed, and suspended in ice water as described by Lieberman and Kornberg (6). Cultures were similarly grown and prepared as above in the absence of OA, glucose replacing OA as a carbon source. Extracts were prepared by treatment in a 10 kc. Raytheon magnetostriction (sonic) oscillator for 20 minutes in the presence of 200 mg. of glass powder. The extracts were centrifuged for 15 minutes at

10,000 × g, and protein determinations on the supernatant solution were made by the Folin method (20) standardized against the bacterial extract by biuret assays. All preparation and storage of the cell-free extracts were carried out near 0°. Storage for long periods of time was at −10°.

Cell-free extracts of *E. coli* were also prepared in the sonic oscillator. For comparison with *Z. oroticum*, the conditions used were identical with those reported above. For other experiments extracts were prepared by sonic oscillation of cells suspended in ice water at a concentration of 2.5 × 10^{10} cells per ml. for 2.0 minutes. The extracts were centrifuged for 5 minutes at 9000 × g to remove whole cells and cellular débris.

Bacterial Counting Methods—The number of *E. coli* cells per ml. of culture was determined either with use of a Petroff-Hausser bacterial counter or by means of turbidity readings (corrected to actual bacterial count) in a Klett-Summerson photometer with a green filter (No. 54). 1 Klett unit is equivalent to 0.9 × 10^7 cells per ml.

Assay for Pyrimidine Intermediates—As a qualitative and semiquantitative test for the presence of the various intermediates, paper chromatography was carried out. In a butanol-ethanol-formic acid-water system (by volume 5:3:2:1) (21), for OA R_F = 0.42, for DHO R_F = 0.45, and for US R_F = 0.53 in descending chromatograms. OA spots were detected by ultraviolet absorption and US and DHO spots by color procedures by using the Ehrlich reagent (22).

Quantitative determinations of OA were made spectrophotometrically at various pH values (23). For the most accurate results, reaction and control samples were passed through Dowex 50 (acid form) columns to remove amine-containing contaminants and were read at 270, 280, and 290 mμ. Orotic acid can be quantitatively recovered in the eluate from the column.

Ureidosuccinate was assayed quantitatively by the colorimetric method of Koritz and Cohen (24). Dowex 50 columns were used as above to remove traces of amine-containing ureido compounds.

Dihydroorotate was assayed by taking advantage of the large loss of optical density (maximal at 230 mμ) found to occur upon alkaline hydrolysis of DHO to US. The rate of hydrolysis is first order with respect to DHO at any given alkali concentration (25). In general, 1.5 ml. of 1 N sodium hydroxide were added to 1.5 ml. of sample in silica Beckman cells at room temperature, mixed rapidly, and read at 240 mμ for 15 minutes at timed intervals. The final equilibrium optical density was subtracted from all values, and the semilog plot of corrected optical density was extrapolated linearly to zero time. No substances in the assay mixtures except DHO changed their optical density significantly in 15 minutes. Both optical extinction coefficient (ϵ) and hydrolysis rate constant

(k_r) vary as alkali concentration is changed, but both reach a plateau and remain fairly constant over an alkali concentration range of 0.2 to 0.8 M NaOH, making the assay essentially independent of buffer concentration and of acid or CO_2 produced by the bacteria. In this plateau range, at 25°, $\epsilon = 6.15 \times 10^3$ mole^{-1}, and $k_r = 0.17$ min.$^{-1}$. A mechanism for the reaction will be proposed elsewhere.

EXPERIMENTAL

Study of E. coli Mutants—In this study of pyrimidine biosynthesis in *E. coli*, two pyrimidine-requiring mutants, as well as wild type *E. coli*, strain B, were used. An effort was made to characterize the point at which biosynthesis was blocked and to determine the rates of utilization or production of pyrimidines or intermediates. These data were later correlated with those obtained from enzyme studies with cell-free extracts.

Preliminary studies showed that mutant 550-460 grew in minimal media containing uracil, uridine, or cytosine, but not thymine, OA, or DHO as growth factors. In the absence of growth factor, this mutant produced large amounts of orotic acid, which could be precipitated from concentrated bacterial supernatant solutions as the potassium salt (26, 7). After purification by charcoal, conversion to the acid form, and recrystallization from water, the bacterial product and commercial orotic acid had identical absorption spectra at all pH values (23), identical titration pK_a values and equivalent weight, identical melting points, showed no depression of a mixed melting point, and both behaved identically on chromatograms (see "Materials and methods").

Although not isolated, US and DHO have been shown to be present along with OA in minimal supernatant solutions of this organism. As shown in Fig. 1, the typical ratio of OA:DHO:US was about 15:1:6. The rate of production of OA + DHO + US varied slightly with conditions of growth, but was about 11×10^{-17} mole per cell per hour in minimal media. In one division time (1.0 hour) 6.7×10^{-17} mole per cell of uracil was taken up by the mutant and a similar amount of nucleic acid pyrimidine was formed.

In contrast to mutant 550-460, mutant 6386 was observed to use OA as well as uracil for a growth factor, but not to use DHO or US. Several substrains of this mutant could be differentiated by their growth rates with OA. The division times observed were 1.1, 1.8, 3.3, 6.8, and 10 (or more) hours.

As expected, no OA was produced by suspensions of this mutant in minimal medium; however, DHO was present. The DHO was isolated from one minimal supernatant solution containing only 5 per cent the normal salt concentration by chromatography on Dowex 1 (formate), fol-

lowed by removal of cations by Dowex 50 (acid form) resin and vacuum concentration to dryness (6). After three recrystallizations from water, the bacterial product and synthetic L-dihydroorotic acid had the same melting point (274° (decomposed)) with no depression of a mixed melting point, the same optical rotation, the same extinction coefficient and hydrolysis

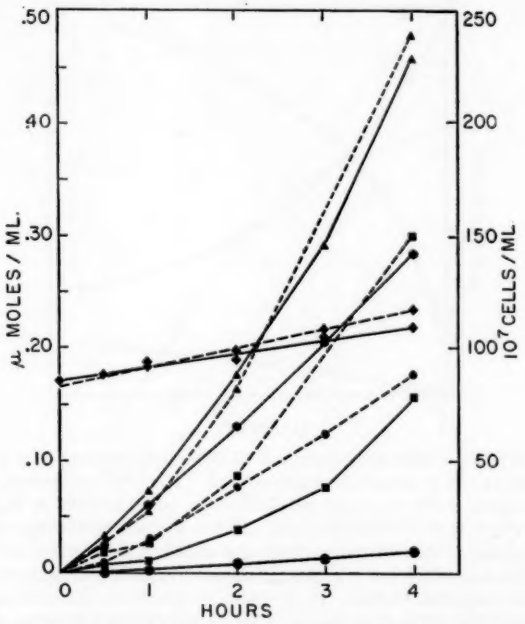

FIG. 1. Production of pyrimidine intermediates by *E. coli* mutants. See the text for details. The dotted lines are used for mutant 6386 and the solid lines for mutant 550-460. ●, dihydroorotic acid production; ■, ureidosuccinic acid production; ⧫, orotic acid production; ▲, total production of pyrimidine intermediates; ◆, cell concentration of mutant 6386 and of mutant 550-460.

rate constant in alkali, and identical chromatographic properties with the solvent mixture used above for OA.

As shown in Fig. 1, US as well as DHO was produced by this mutant when suspended in minimal medium. As with mutant 550-460, the rates of US and DHO production varied slightly with conditions used, but under the same conditions reported for mutant 550-460, mutant 6386 produced US and DHO in a ratio of 1.4:1 and total production of 11×10^{-17} mole per cell per hour. Also, in one division time (1 hour) 6.7×10^{-17} mole

per cell of added uracil or OA was consumed by the growing cells (*i.e.*, 1 μmole allowed production of 1.5×10^{10} cells).

Studies on Cell-Free Extracts—Z. oroticum cells were grown and cell-free extracts were prepared as described under "Materials and methods." For the study of DHO oxidation, the reaction mixtures were read in a Beckman

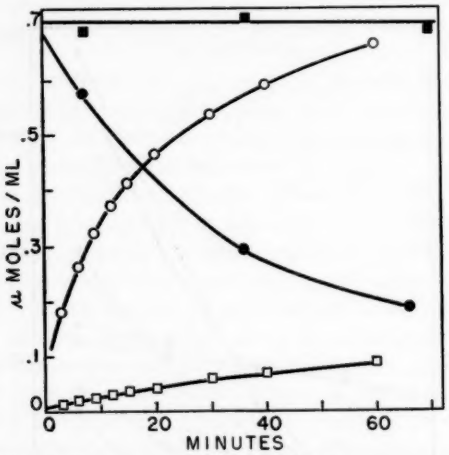

FIG. 2. Activities of dihydroorotase and of DHO dehydrogenase by extracts of *Z. oroticum* grown in the presence or absence of OA. For DHO oxidation, the reaction mixtures contained 0.033 M potassium phosphate, pH 6.5, 0.005 M $MgCl_2$, 0.005 M cysteine, pH 7.0, 1.33×10^{-4} M DHO, and cell-free extract of 0.50 mg. of protein per ml. Total volume 3.0 ml. The substrate was added at zero minute to the reaction mixture and readings were made at 290 mμ against controls containing no substrate. OA values are magnified 10-fold. ○, *Z. oroticum* grown on OA enrichment media; □, *Z. oroticum* grown on glucose media. For study of DHO hydrolysis, the reaction mixtures contained 0.033 M potassium phosphate, pH 6.5, 0.005 M $MgCl_2$, 0.0017 M Versene, 7×10^{-4} M DHO, and cell-free extract of 0.50 mg. of protein per ml. Total volume 6.0 ml.; 37°; aliquots assayed at intervals for DHO as described under "Materials and methods." ●, *Z. oroticum* grown on OA enrichment media; ■, *Z. oroticum* grown on glucose medium.

DU spectrophotometer at 290 mμ against control samples lacking DHO. Fig. 2 shows that the initial rate of OA formation by extracts of *Z. oroticum* grown on OA medium was quite rapid, while extracts of *Z. oroticum* grown on glucose medium in the absence of OA had only about 5 per cent as much activity. As also demonstrated in Fig. 2, the activity of a second enzyme, dihydroorotase, was quite pronounced in extracts of *Z. oroticum* grown on OA, while extracts of the organism grown in the absence of OA were essentially inactive.

The extremely low activities of both these enzymes in *Z. oroticum* grown

on glucose compared to the activities of cells grown in the presence of OA (Fig. 2) indicate that the enzymes may have been formed adaptively on exposure of the cells to OA. The enzymes might have been formed only

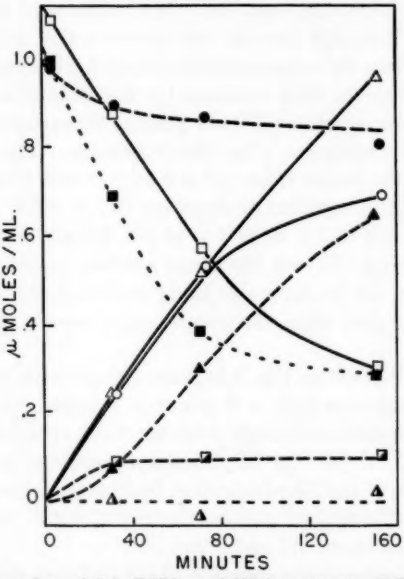

FIG. 3. Interconversion of OA, DHO, and US by extracts of *E. coli*, strain B, and mutant 6386. Conditions, 0.033 M potassium phosphate, pH 6.5, 0.025 M $MgCl_2$, 0.001 M Versene, and 0.42 mg. of extract protein (2.4×10^9 cells) per ml. The total volume was 6.0 ml. At zero time, substrate (L-DHO or DL-US) was added to 0.001 M concentration, mixed, and incubated at 37°. Samples were removed at intervals and assayed qualitatively and quantitatively for OA, DHO, and US as described in the text. The control samples lacked substrate. The values on the graph are concentrations in complete reaction mixtures minus values in control samples. OA values are magnified 10-fold. For *E. coli*, strain B, extract, DHO substrate plots are solid lines; □ = DHO; △ = OA; ○ = US. DL-US substrate plots are dash lines; ● = US; ◪ = DHO; ▲ = OA. For mutant 6386 extract, DHO substrate plots are dotted lines; ■ = DHO; ▲ = OA.

to use OA as a carbon source for metabolism and not to use it as a pyrimidine precursor. The low but noticeable activity of the dehydrogenase in extracts of *Z. oroticum* grown on glucose media, however, might have been enough to account for the observed slow growth rate of the cells. Since quantitative determinations are difficult at the above low levels of activity, results obtained with cell-free extracts of the faster growing organism, *E. coli*, strain B, grown on minimal medium may be more pertinent.

Fig. 3 presents results with extracts of *E. coli*, strain B, grown on minimal medium and extracts of mutant 6386 grown on this medium plus uracil. As can be seen, the *E. coli* extracts oxidized DHO to OA and converted DHO to US and US to DHO readily, with good stoichiometry, since only the L isomer of DL-US was used (6). The extracts of mutant 6386 converted DHO to US readily but did not form any OA, indicating that mutant strain 6386 lacks the enzyme dihydroorotic acid dehydrogenase. This finding fits well with the data obtained for the mutant *in vivo*.

As mentioned above, either DHO or US was converted to an equilibrium mixture of the two compounds by dihydroorotase. The equilibrium was determined over the range from pH 5.9 to 8.0 and found to be pH-dependent. At 37° the equilibrium constant [$K_e = ((US^-)(H^+))/(DHO^-)$] had an average value of 1.4×10^{-6} M at pH 6.0 and showed a slight decrease with increasing pH over the range studied (at pH 8.0, $K_e = 0.6 \times 10^{-6}$). This value for Ke compares fairly well with the value 1.5×10^{-6} M at pH 6.1 calculated from the experimental results of Lieberman and Kornberg (6).

Although the results from Fig. 3 indicate the presence of dihydroorotase and DHO dehydrogenase in *E. coli* grown in minimal medium, the conditions used for the determinations were far from optimal. Fig. 4 shows that, for hydrolysis of DHO to US, the dihydroorotase has a pH optimum of 8.0. The shape of the dihydroorotase hydrolytic activity curve may be attributed to the titration of two groups in the active site of the enzyme (27) with pK values about 7.7 and 8.2 at 36°.

The optimal pH range for conversion of US to DHO is quite broad, with an optimum at about pH 6. The dihydroorotase activity for DHO formation cannot be determined with any accuracy above pH 7.2, since the equilibrium ratio of US:DHO is quite unfavorable, but the pK of the active site of the enzyme is about 7.6, the same as that found above.

When enzyme reaction rates were studied as a function of substrate concentration and the results were plotted according to Lineweaver and Burk (28), the results in Fig. 5 were obtained. For conversion of US to DHO at pH 5.9, the Michaelis constant (K_s) = 5.3×10^{-4} M L-US, and maximal velocity (Vm) = 11×10^{-16} mole per cell per hour, far above that required by cellular need for pyrimidines. Orotic acid is a competitive inhibitor in this system with an inhibition constant, $K_i = 1.3 \times 10^{-3}$ M. It was found also that the activation energy for this conversion was 10,600 calories.

The conversion of DHO to US at pH 8.0 has $K_s = 3 \times 10^{-4}$ M and $Vm = 18 \times 10^{-16}$ mole per cell per hour. The maximal velocity varies somewhat with different extracts and appears to require an unknown cofactor (not magnesium or calcium ions) at low extract concentrations. The activation energy for this conversion was found to be about 12,000 calories.

The dihydroorotic acid dehydrogenase from *E. coli* was also studied. The pH optimum for DHO oxidation is quite broad with a pK about 8.0,

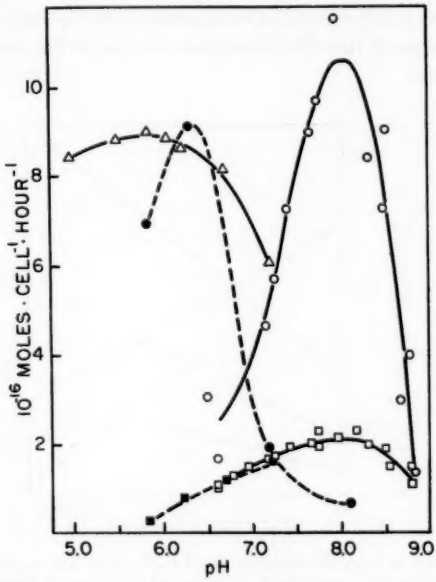

FIG. 4. Enzyme activities as a function of pH. All experiments were carried out at 37° with an *E. coli*, strain B, extract of 2.5×10^9 cells per ml. (0.46 mg. of protein per ml.). For ureidosuccinic acid synthetase (●), the reaction mixtures contained 0.1 M potassium phosphate buffer, or 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer above pH 8, 0.01 M carbamyl phosphate, 2×10^{-4} M L-aspartic acid-4-C^{14}, and 5×10^{-3} M magnesium chloride. The observed value at pH 6.2 was corrected to the calculated rate at saturating substrate concentrations, and other points were adjusted proportionately. The curve is thus only approximately correct. With DHO as substrate, dihydroorotase (○) and dihydroorotic acid dehydrogenase (□) reaction mixtures contained 0.1 M Tris buffer, 0.001 M Versene, and 0.002 M DHO. The total volume was 5.0 ml. At timed intervals samples were passed rapidly through Dowex 50 (acid form) columns and assayed as described in the text. Initial reaction rates were calculated and plotted on the graph. With US as substrate, dihydroorotase (△) and dihydroorotic acid dehydrogenase (■) reaction mixtures contained 5×10^{-4} M Versene, 4×10^{-3} M DL-US, 0.1 M potassium phosphate buffer above pH 5.8, and acetic acid plus 0.1 M sodium acetate as buffer below pH 5.6. Other conditions as described above.

as shown in Fig. 4. At pH 7.25, Fig. 5 shows that $K_s = 4 \times 10^{-5}$ M DHO and $Vm = 1.6 \times 10^{-16}$ mole per cell per hour, a rate more than fast enough to account for observed pyrimidine biosynthesis in whole cells. The Michaelis constant was determined by using an equilibrium mixture

of US and DHO as substrate, as calculated from the pH and K_s. The dihydroorotase in the extract, therefore, helped to keep the amount of DHO constant. From the relative shapes of the curves, the rate of production of OA from US is seen to be dependent on the rate of the dehydrogenase rather than of the dihydroorotase, as might be expected from the relative K_s values.

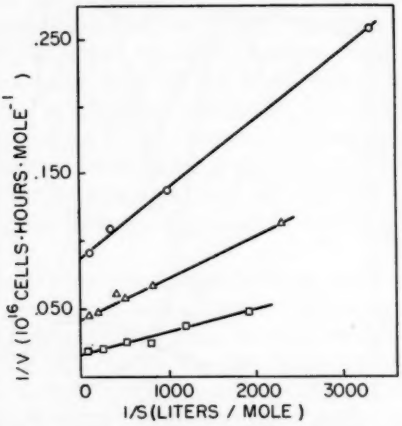

FIG. 5. Variations in enzyme reaction rates with substrate concentrations. Conditions were the same as those in Fig. 4 except as noted below. With US as substrate, dihydroorotase (○) reaction mixtures contained 0.1 M potassium phosphate, pH 6.0, 0.002 M Versene, and DL-US (calculated as L-US). The total volume was 5.0 ml. With DHO as substrate (□) the reaction mixtures contained 0.1 M Tris buffer, pH 7.8 (adjusted to 0.1 ionic strength with KCl), 0.001 M Versene, varied DHO, and extract of mutant 6386. *Note* scale of graph changed: Observed $1/v$ and $1/s$ both plotted as one-fourth actual values. For dihydroorotic acid dehydrogenase (△) the reaction mixture contained 0.1 M potassium phosphate, pH 7.4, mixture of L-DHO and DL-US in the ratio 1:50 (L-DHO:L-US of 1:25). Observed $1/s$ and $1/v$ both plotted as one-twentieth actual values.

A study was made also on the enzymatic synthesis of US from carbamyl phosphate and aspartate, first reported by Jones *et al.* (1). The method employed was to incubate *E. coli* extracts with carbamyl phosphate and C^{14}-aspartic acid under various conditions, then pass the reaction mixtures quantitatively through small columns of Dowex 50 (acid form) to adsorb the aspartic acid. Aliquots of the eluate were counted directly for C^{14} activity, or isotope dilution experiments with non-radioactive, synthetic DL-US were performed.

Each method of assay has reasons to recommend it. Direct plating of

the eluate is the easier method, allows more sensitive determination of radioactivity, and assays total reaction rate (formation of US + DHO + OA) over a given interval, but must be corrected for the radioactivity from products of aspartic acid deamination. Although isotope dilution is a less sensitive method and only US is determined by it, the need for the above correction is avoided.

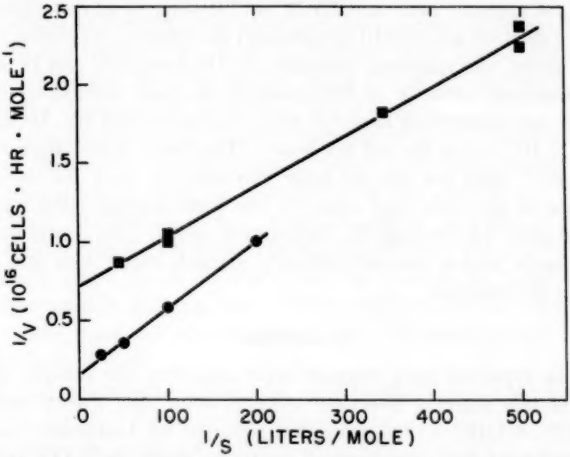

FIG. 6. Variation of reaction rate of ureidosuccinic acid synthetase with substrate concentration. The incubation mixture contained 0.1 M potassium phosphate, pH 6.5, 0.005 M $MgCl_2$, 0.010 M C^{14}-DL-aspartic acid unless varied (results plotted as L-aspartic acid), 0.010 M carbamyl phosphate unless varied, and cell-free extract of 2.5×10^9 *E. coli* cells (0.44 mg. of protein). Total volume 1.0 ml. The reaction mixtures were incubated at 37° for 1.5 hours, rinsed through Dowex 50 (acid form) columns, plated, and counted directly in a gas flow Geiger-Müller counter. Corrections were made for control samples as described in the text. ●, variable aspartic acid concentration; ■, variable carbamyl phosphate concentration.

The non-enzymatic formation of US, first order with respect to both aspartate and carbamyl phosphate concentrations, also requires a correction. At 0.03 M concentrations of each compound, US can be formed non-enzymatically at a rate of 0.48 μmole per ml. per hour, a rate faster than the enzymatic rate at the usual extract concentration of 2.5×10^9 cells per ml. To avoid this large non-enzymatic reaction, enzyme rates were usually determined at much lower substrate concentrations.

The rate of formation of US by the extracts was found to be fairly constant over a period of 2 hours with a slight decrease in rate beyond that period. The enzyme was completely inactivated by heating for 2 minutes

at 100°, and addition of inactivated extract to the reaction mixtures had no stimulatory effect. Magnesium ion did not activate the reaction, which proceeded at an optimal rate even in 0.2 M NaF, but instead became inhibitory above concentrations of 0.01 M.

When the rate of US synthesis was studied as a function of aspartate and of carbamyl phosphate concentration, the results shown in Fig. 6 were obtained. K_s of carbamyl phosphate was calculated to be 4.3×10^{-3} M, and K_s of aspartic acid was 2.5×10^{-2} M (Fig. 6) at concentrations of 0.005 M L-aspartate and of 0.01 M carbamyl phosphate, respectively. From the same plots, the maximal velocities of US synthesis can be obtained, and the maximal velocity of US synthesis at both saturating carbamyl phosphate and saturating aspartic acid concentrations can be calculated to be 9.1×10^{-16} mole per cell per hour. This value is far larger than that of 6.7×10^{-17} mole per cell per hour necessary to allow for the observed growth rate of the cells, and need for the high enzyme level may be explained, in part, by the high K_s for aspartic acid. The actual concentration of aspartic acid in the cell probably is much lower than that required to saturate the enzyme.

DISCUSSION

The work reported here supports and amplifies the scheme shown at the start of this paper. The two enzymes catalyzing the reversible conversions US $\rightleftharpoons$ DHO $\rightleftharpoons$ OA were first reported by Lieberman and Kornberg to be present in an organism, *Z. oroticum*, grown with OA as a carbon source. As shown here, however, *Z. oroticum* cultured in glucose medium grew faster than it did when OA was used, yet contained only small amounts of the two enzymes. This result indicated the adaptive formation of the enzymes to metabolize OA for general metabolic purposes and raised the question of whether these enzymes were involved in pyrimidine biosynthesis. The studies with *E. coli in vitro*, however, showed activities of ureidosuccinic acid synthetase, dihydroorotase, and dihydroorotic acid dehydrogenase more than enough to account for the growth rates of the whole cells. The low activities of the enzymes in *Z. oroticum* grown in glucose medium were probably adequate for the low growth rate of the cells, however, although quantitative study was difficult.

Since one substrain of *E. coli* mutant 6386, which lacks the enzyme dihydroorotic acid dehydrogenase, was able to grow on OA at the normal rate for wild type cells, attachment of ribose (9–11) at a step prior to OA formation was not necessary to account for pyrimidine formation. Summation of the work *in vivo* and *in vitro* shows that the scheme at the start of this paper is valid for *E. coli* and can explain the total observed growth rate of the cells.

SUMMARY

1. One pyrimidine-requiring *Escherichia coli* mutant has been characterized as lacking the enzyme dihydroorotic acid dehydrogenase. This mutant does not produce orotic acid, but can use it as a growth factor. Another mutant uses uracil and cytosine as growth factors and excretes ureidosuccinic acid (US), dihydroorotic acid (DHO), and orotic acid (OA) in the absence of pyrimidine growth factors.

2. The rates of production of total pyrimidine intermediates by the two mutants in the absence of uracil or cytosine were nearly identical and were slightly greater than the rate of utilization of pyrimidine for optimal nucleic acid formation and growth of the culture.

3. Cell-free extracts of *Zymobacterium oroticum* grown in glucose medium had about one-twentieth the dihydroorotase and dihydroorotic dehydrogenase activities of extracts of cells grown in OA medium.

4. Cell-free extracts of *E. coli*, strain B, grown in minimal media converted aspartic acid plus carbamyl phosphate to US, US to DHO, and DHO to OA several times faster than necessary to account for observed pyrimidine biosynthesis in growing cells. This evidence, plus the results *in vivo*, indicates that these conversions are part of the pathway for pyrimidine biosynthesis in *E. coli*.

5. pH optima, substrate affinities, and maximal rates of reaction were determined for the above conversions.

6. A convenient assay for DHO is reported.

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CONTROL OF PYRIMIDINE BIOSYNTHESIS IN ESCHERICHIA COLI BY A FEED-BACK MECHANISM*

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The mechanisms by which a cell can control its biosynthetic processes are important from the point of cellular economy, yet they are little understood. It is known that bacteria must have such control systems, since their biochemical processes are so well coordinated, as shown by failure of the cell to accumulate large amounts of metabolites in the medium (1).

Several indications of control mechanisms have been found. One type of observation is that addition of a few micrograms per ml. of normal purines (2), pyrimidines (1), or amino acids (3, 1) to growth media of normal bacteria could result in nearly exclusive incorporation of these materials into cell constituents, in preference to *de novo* synthesis of the materials by the cell. It has been noticed also that production of intermediates of the above materials can be inhibited by addition of the end-products to cultures of suitable bacterial mutants. Gots and Chu (4) and Love and Gots (5) found that addition of purines to the culture medium of purine-requiring *Escherichia coli* mutants caused inhibition of formation of two purine precursors, and Brooke *et al.* (6) showed the inhibition of orotic acid (OA)[1] formation in the presence of uracil by pyrimidine-requiring *Aerobacter aerogenes* mutants. Adelberg and Umbarger (7) observed that the presence of the growth factor, valine, inhibited formation in an *E. coli* mutant of α-ketoisovalerate, a valine precursor, and commented that the unknown mechanism was undoubtedly wide-spread and important to the economy of the cell.

Work from this Laboratory indicated that the presence of uracil inhibited formation of pyrimidine intermediates earlier than orotic acid in pyrimidine-requiring *E. coli* mutants (8). It seemed of interest to determine whether this observation plus the above facts could be the results of normal self-regulatory mechanisms of the cell which allowed formation of no more

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† Abraham Rosenberg Research Fellow during the course of this work.

[1] The abbreviations used are as follows: OA = orotic acid (4-carboxyuracil); DHO = dihydroorotic acid; US = ureidosuccinic acid (carbamylaspartic acid); RNA = ribonucleic acid; DNA = deoxyribonucleic acid.

components than necessary for growth. For this reason studies *in vivo* and *in vitro* on the control of pyrimidine biosynthesis in *E. coli*, strain B, and two pyrimidine-requiring mutants of *E. coli* were carried out.

Materials and Methods

Uracil, cytosine, uridine, orotic acid, cytidine-5′-phosphate, and 4-C^{14}-DL-aspartic acid were obtained commercially. Cytidine was kindly provided by Dr. C. A. Knight and uridine-5′-phosphate by Dr. C. Dekker of these laboratories. DL-US and L-DHO were prepared as described in the accompanying paper (8). *E. coli* strain B and the two pyrimidine-requiring *E. coli* mutants (6386 and 550-460) were obtained and cultured in the minimal medium (containing salts and glycerol) described earlier, with uracil or OA as growth factor when needed (8). For inactivation of nucleic acid formation in the bacteria, freshly grown cells were washed, suspended in minimal salt solution at a concentration of 2.5×10^{10} cells per ml., and irradiated for 3 minutes in a large Petri dish (suspension 0.5 cm. deep) 14 inches below three Westinghouse sterile lamps No. WL-782L-30. Under the conditions used, both DNA and RNA syntheses by the cells were essentially abolished and fewer than 0.001 per cent of the cells were capable of colony formations on broth plates.

EXPERIMENTAL

Control in Vivo of Pyrimidine Intermediate Production by Mutants—In the study of pyrimidine-requiring mutant 550-460 it was noticed that large amounts of the pyrimidine intermediates US, DHO, and OA were produced only in media free of the pyrimidines uracil, cytosine, or their nucleosides or nucleotides (8). Under similar conditions mutant 6386 produced US and DHO only in the absence of the above pyrimidines. Thymine neither stimulated growth nor inhibited production of the intermediates. The effect of traces of uracil in this inhibition can be seen in Fig. 1, where production of the intermediates became significant only when the added uracil had been exhausted by cell growth. The above results extend the report by Brooke *et al.* (6) that OA formation was inhibited by the presence of uracil in pyrimidine-requiring *A. aerogenes* mutants.

It was necessary to determine whether the inhibition was a direct effect of the pyrimidine or was dependent in some way on cell multiplication, since under the above conditions cells were growing whenever the pyrimidine growth factor was added to the cultures. A substrain of *E. coli* mutant 6386, characterized by rapid growth with OA as a growth factor, was isolated. As shown in Fig. 2, the rate of growth was comparable in minimal medium containing OA or uracil, yet the rate of DHO production

per cell in OA-containing medium was even higher than production by cells suspended in minimal medium. This result indicated that the inhibition was specifically brought about by uracil. Apparently the uptake and conversion of OA to uridylic acid (9, 10) were rate-limiting for growth.

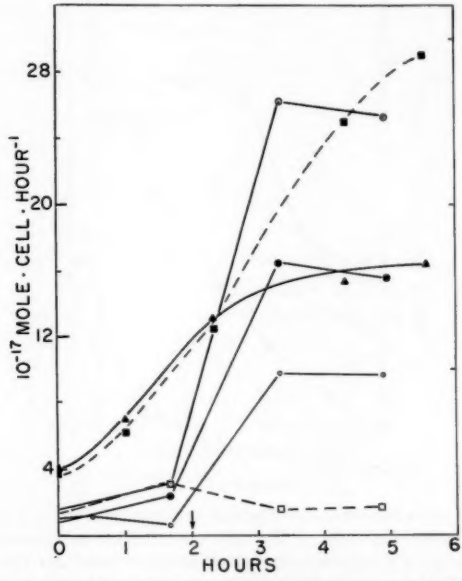

FIG. 1. Rate of production of US and DHO by *E. coli* mutant 6386 and dependence on concentration of uracil in the medium. See the text for details. ■ and ▲, cell count of culture containing initial concentrations, respectively, of 2.0×10^{-4} M and 5.0×10^{-5} M uracil. □, total rate of US plus DHO production in culture containing 2.0×10^{-4} M uracil. ○, ●, and ⊙, rates of production of DHO, US, and of DHO plus US, respectively, in culture containing 5.0×10^{-5} M uracil. Initial concentration of 1.0×10^{-4} M uracil allows formation of 1.5×10^{9} cells. The arrow indicates when the 5.0×10^{-5} M uracil should be exhausted.

Thus the inhibitory compound was used for nucleic acid synthesis as rapidly as it was formed from orotic acid and did not accumulate inside the cell.

If the above ideas are correct, inhibition of nucleic acid biosynthesis at a stage beyond the formation of nucleotides should result in inhibition of formation of the pyrimidine intermediates. To test this, washed mutant 6386 cells, freshly grown in minimal medium with OA as growth factor, were irradiated heavily with ultraviolet light to halt both DNA and RNA biosynthesis. These cells were then incubated in minimal medium and in

medium plus OA. In minimal medium the rate of DHO production per irradiated cell decreased slowly with time, presumably partly as a result of enzyme destruction (Fig. 3). Irradiated cells in medium containing OA, however, very soon showed a decrease in the rate of DHO forma-

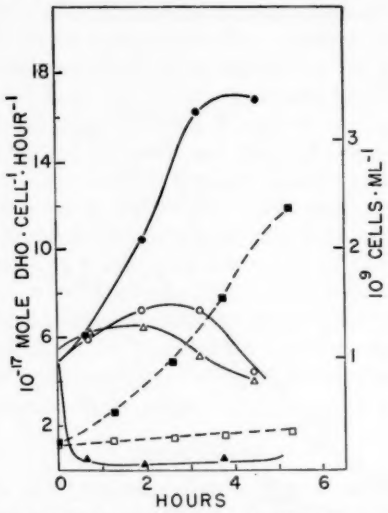

FIG. 2. Rates of pyrimidine intermediate production by growing and by non-growing mutant 6386 cells. Cultures of *E. coli* mutant 6386 cells grown on minimal medium plus OA or plus uracil were harvested and incubated for 1 hour in minimal media. Reharvested and washed, the cells were suspended in minimal medium alone or in media containing the same growth factor used before. □ and ■, cell count (turbidity) of cells suspended, respectively, in minimal media and in media containing 2×10^{-4} M uracil or orotic acid (growth rates nearly identical). △ and ▲, per cell rate of production of DHO by cells previously grown in media containing uracil and resuspended in minimal medium and in medium containing 2×10^{-4} M uracil, respectively. ○ and ●, per cell rate of production of DHO by cells previously grown in media containing OA and resuspended in minimal medium and in medium containing 2×10^{-4} M OA, respectively.

tion, the decrease being almost as rapid as that caused by the presence of uracil in the medium. The rate of inhibition by growth factor appears to be correlated with normal growth rates, since cells grew slightly faster with uracil than with OA. The results support the idea that when a cell does not use a uracil derivative as rapidly as it is produced from OA, this derivative accumulates and inhibits the formation of the pyrimidine intermediates US and DHO.

Estimation in Vivo of Inhibition Constant—The concentration of uracil

derivative in the cell necessary to cause half inhibition of the rate of DHO production was determined. The ability of ultraviolet-irradiated 6386 cells to remove uracil from the medium was measured by bioassay with mutant 550-460 (which requires uracil for growth). About 6.7×10^{-17}

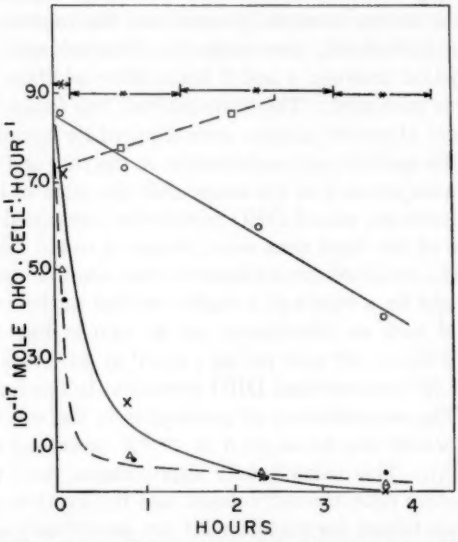

FIG. 3. Pyrimidine intermediate production by ultraviolet-irradiated mutant 6386. The cells were grown at 37° in minimal medium containing 2.0×10^{-4} M OA (division time 2.0 hours) and samples taken at intervals were assayed for DHO. The culture was centrifuged, washed, and suspended in minimal salts, then irradiated with ultraviolet light for 2 minutes and suspended at about 6.0×10^8 cells per ml. of test medium. Samples were removed at intervals and tested for DHO. The average amount of DHO produced per hour per bacterium over the time intervals shown at the top of the graph is represented by the area under a plotted line. □, growing cells on minimal medium plus 2.0×10^{-4} M OA. ○, ultraviolet-treated cells on minimal medium. ×, ultraviolet-treated cells on minimal medium plus 2.0×10^{-4} M OA. △, ultraviolet-treated cells on minimal medium plus 1.0×10^{-5} M uracil. ●, ultraviolet-treated cells on minimal medium plus 1.0×10^{-4} M uracil.

mole of uracil could be removed per irradiated cell. If the media contained less than this amount of uracil, the uracil could be quantitatively removed from the medium.

The amount of uracil necessary to cause 50 per cent inhibition of pyrimidine formation by a cell was determined by experiments in which various amounts of uracil were added to minimal media containing ultraviolet-irradiated 6386 cells. The rates of DHO production by the cells were

then measured. The specific conditions were as follows: Cells were first grown in minimal medium plus uracil, harvested, washed, and incubated for 1 hour in minimal medium to allow exhaustion of any "free" pyrimidine in the cell. The cells were again harvested, washed, suspended in minimal salt solution, and ultraviolet-irradiated. Aliquots were then added to media containing known amounts of uracil and the concentrations of cells were determined by turbidity measurement. Determination of the amount of DHO production between 1 and 2 hours after addition of cells to the uracil media was measured. This time interval was found to be the most satisfactory, since about 40 minutes were required for complete absorption of uracil from the medium and stabilization of the rate of DHO formation by the cells. Also, about 2 to 2.5 hours after the start of the experiment the markedly decreased rate of DHO production mentioned above became apparent, while at the same time some release of uracil inhibition caused an increase in the inhibited rates relative to the non-inhibited ones. The latter effect might be a result of a slight residual nucleic acid synthesis.

The results of such an experiment can be seen in Fig. 4, where, at a concentration of 6.2×10^8 cells per ml., uracil at an initial concentration of about 3.4×10^{-6} M depressed DHO formation to one-half of the uninhibited rate. The concentration of pyrimidine in the cell to give 50 per cent inhibition would thus be about 5×10^{-3} M (assuming a unit cell volume of 10^{-12} ml.). This value is only approximate, since the number of metabolically active cells, the cell volume, and the fraction of uracil in the cell in a form not bound (as nucleic acid) are known only approximately. Further, the actual inhibitor might be a free base, riboside, ribotide, or deoxyribotide of either uracil or cytosine. The ratio of these forms in the cell and also the concentration of substrate at the site of inhibition are unknown; hence no value can be calculated for an enzyme inhibition constant. The value found might be expected to agree within a factor of 10 with the true inhibition constant, however.

Inhibition in Vitro of Ureidosuccinic Synthetase—The studies *in vivo* had shown that the inhibitor appeared to act at or before the step of biosynthesis of US, and that later interconversions of pyrimidine intermediates were not affected by the inhibitor. Further, US has been shown to be formed enzymatically from aspartic acid and carbamyl phosphate (11, 8), and it was felt that synthesis of neither the aspartic acid nor the carbamyl phosphate was the site of action of the inhibitor. This feeling was held because carbamyl phosphate, which appears to be necessary for arginine biosynthesis (11), and aspartic acid would both be necessary for protein formation, yet there was no inhibition of protein synthesis when the mutant was grown with uracil. Under such conditions US, DHO, and OA were not produced. Therefore, it appeared likely that the site of the inhibition would be the formation of US itself.

Experiments *in vitro* were carried out as described (8) to determine the rates of US formation under the influence of various possible inhibitors. As shown in Table I, uracil, uridine, uridine-5′-phosphate, and cytosine had no observed inhibitory effects. Cytidine and especially cytidine-5′-phosphate, however, had quite large inhibitory effects. The inhibition appears to be competitive with respect to both carbamyl phosphate and aspartic acid. Against carbamyl phosphate, the inhibition constant, K_i,

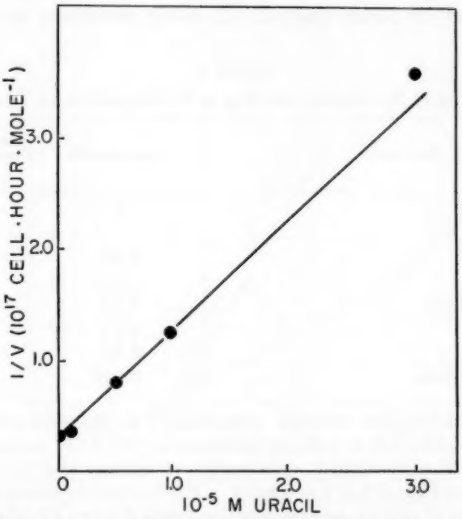

FIG. 4. Uracil inhibition of DHO production by ultraviolet-irradiated cells of *E. coli* 6386. Details in the text. *E. coli* mutant 6386 cells ultraviolet-irradiated for 3.0 minutes were suspended at 6.2×10^8 cells per ml. in minimal medium containing uracil at the concentration shown. The inverse of the per cell rate of DHO production between 1.0 and 2.0 hours after start of incubation is plotted in the graph.

of cytidylic acid was 6×10^{-3} M, and K_i of cytidine was about 8×10^{-3} M (Fig. 5). The results with cytidine as an inhibitor were rather variable, however. It would be reasonable to suspect that cytidine-5′-phosphate (or a derivative) is the true inhibitor *in vivo*, for it is a more effective inhibitor and also might be expected to remain in the cell owing to its negative charge. The reaction appeared to be only slightly competitive with respect to aspartic acid concentration ($K_i \sim 0.01$ M), but the fraction of release of inhibition by either substrate cannot be determined very accurately. The effect of phosphate ion on the reaction is also unknown.

Metabolic Studies—With the finding that an end-product of biosynthesis apparently inhibited the initial step of a whole pathway, it became of in-

terest to determine how wide-spread this phenomenon might be. It has been noticed that when the pyrimidine-requiring mutants exhaust their pyrimidine growth factor, growth, protein synthesis, and nucleic acid synthesis are almost completely stopped (12), but, as shown here, production of the pyrimidine intermediates continues at the rate normal for pyrimidine synthesis of growing, wild type cells. If synthesis of many other metabolites continued at the same rate as in growing cells, the fraction of carbon and nitrogen from the medium converted to the pyrimidine intermediates should be about normal. If other syntheses in the cell were

TABLE I

Effect of Pyrimidines on Rate of US Formation in Vitro

Inhibitor	Concentration	Rate of US formation (moles per hr. per 10^{16} cells)
	M	
None		0.96
Uracil	0.04	0.96
Uridine	0.02	0.88
Uridine-5′-phosphate	0.02	1.00
Cytosine	0.04	0.92
Cytidine	0.02	0.60
Cytidine-5′-phosphate	0.015	0.48

Conditions—The reaction mixtures contained 0.1 M potassium phosphate buffer, pH 6.5, 0.005 M $MgCl_2$, 0.01 M carbamyl phosphate, 0.02 M C^{14}-DL-aspartic acid, extract of 2.5×10^9 *E. coli*, strain B, cells per ml. of reaction, and the inhibitor above carefully neutralized to pH 6.5, if necessary. The reaction mixtures were warmed to 37° before addition of extract, and the reactions were stopped by passage of aliquots through Dowex 50 (acid form) columns. Samples were directly plated and counted for C^{14} and corrected for controls as described (8).

abruptly halted when macromolecule formation ceased, however, many times the normal fraction of carbon and nitrogen should be converted to the unblocked synthesis of pyrimidine intermediates.

From the composition of *E. coli* cells (1), it can be calculated that normally about 4 per cent of the ammonia nitrogen of the medium is converted to the ring nitrogens of the nucleic acid pyrimidines. Table II, however, shows that 50 to 55 per cent of the ammonia (*i.e.* about 13 times the normal amount) could be converted to the pyrimidine intermediates shortly after growth of the mutants was halted by removal of uracil. This result indicates that production of most other nitrogenous compounds can be abruptly halted by the cells. Depending on the previous history of the cells, the per cent incorporation of nitrogen into the pyrimidine precursors actually varied somewhat. If the cells were extensively starved of ammonia

before the experiment, the nitrogen incorporation into pyrimidine intermediates was decreased to about 40 per cent.

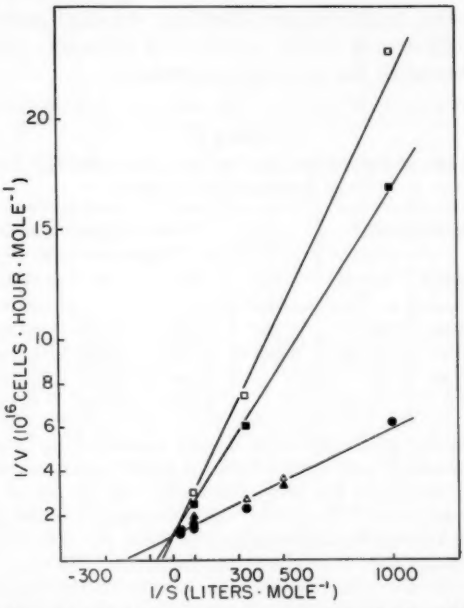

FIG. 5. Rate dependence of US formation on carbamyl phosphate concentration and on inhibitors. The incubation mixture contained 100 μmoles of potassium phosphate buffer, pH 6.5, 5 μmoles of $MgCl_2$, 10 μmoles of C^{14}-DL-aspartic acid, carbamyl phosphate as shown, 20 μmoles of inhibitor (if used), cell-free extract of 2.5×10^9 *E. coli* strain B cells (0.44 mg. of protein) prepared by treatment for 15 minutes in a sonic oscillator. Total volume 1.0 ml. The reaction mixtures were incubated at 37°, and the reaction was halted by passage through Dowex 50 (acid form). The eluate, plus rinse water, was plated, dried, and counted directly in a gas flow Geiger-Müller counter. Correction was made for control samples as described before (8). ●, ▲, and △, no inhibitors (different extracts). □, presence of cytidine-5′-phosphate. ■, presence of cytidine.

If formation of all nitrogenous compounds were self-controlled, it might be expected that 100 per cent of the ammonia would be converted to the non-controlled pyrimidine intermediates, but in practice there will always be slow leakage of all materials from the cell. This leakage would be replaced by synthesis to keep the inhibiting end-product at a high enough concentration to cause further inhibition. Also, the remaining small amount of cell growth (12) would tend to tie up nitrogenous compounds.

Results from the study of carbon incorporation are similar to those with nitrogen, although allowance must be made for oxidation of the carbon source, glycerol, for energy production. The production of pyrimidine intermediates can be determined either by standard chemical assays or by use of C^{14}-glycerol as carbon source with subsequent isotope dilution methods to determine the amounts of products.

TABLE II

Formation of Pyrimidine Intermediates from Limiting Amounts of Ammonia and Glycerol

Mutant	Starting material*		Products in incubation mixture*				
	$(NH_4)_2SO_4$	Glycerol	OA	DHO	US	Total	Fraction incorporated†
550-460	0.30	70	0.100	0.018	0.032	0.150	0.50 (N)
	10.0	1.50	0.080	0.015	0.021	0.116	0.13 (C)
6386	0.30	70		0.083	0.082	0.165	0.55 (N)
	10.0	1.50		0.104	0.076	0.180	0.20 (C)

Conditions—Freshly grown cells were washed, incubated for 45 minutes in minimal medium, harvested, and washed twice in 0.05 M potassium phosphate buffer (pH 7.2) before addition to the test solutions at 1×10^9 per ml. Samples were taken after incubation for 1.5 hours at 37° and centrifuged, and the supernatant solutions were passed through Dowex 50 (acid form) columns. The eluates, diluted 1:1 with the water rinse of the columns, were assayed for OA, DHO, and US as described before (8). The values are the differences between the reaction sample and a control sample lacking the limiting factor in the reaction sample. Samples taken at later incubation times showed no greater total production of intermediates than shown above, although the relative amounts changed.

* All concentrations are $\times 10^{-3}$ M.

† C = carbon; N = ammonia.

Growth of *E. coli* strain B cells in minimal medium containing limiting amounts of glycerol showed that 6.75×10^{-6} mole of glycerol allowed formation of 1.3×10^9 cells containing 8.7×10^{-8} mole of pyrimidine. Thus at the normal rate of pyrimidine biosynthesis, about 2.2 per cent of the glycerol would be expected to pass through the pyrimidine intermediates. It can be calculated further that of the 2.5×10^{-4} gm. per ml. of glycerol carbon, about 1.6×10^{-4} gm. of carbon was incorporated into the cells for a 65 per cent efficiency of carbon utilization.

Tables II and III show that 13 to 20 per cent of the carbon could be converted to the pyrimidine intermediates by the mutant cells. This value is seen to be quite variable, depending on whether the cells were previously starved of glycerol, but is 7 times greater than the 2.2 per cent expected if syntheses of all other carbon compounds continued. The observed value,

however, was far below both the per cent of nitrogen incorporation and the expected limit of 65 per cent carbon incorporation, indicating that synthesis of many carbon compounds, perhaps fats, phospholipides (12), and polysaccharides, still continued in the pyrimidine-deficient cell.

TABLE III

Per Cent Incorporation of C^{14} into US, DHO, and OA by Mutants 6386 and 550-460

Mutant	Condition	Product			
		US	DHO	OA	Total
6386	Non-starved	2.3	8.0		10.3
	Starved of glycerol	2.2	4.6		6.8
550-460	Non-starved	0.81	*	17.3	19
	Starved	0.64	*	11.9	13

Procedure—Freshly grown cells were suspended for 30 minutes at 37° in minimal medium to exhaust the cells of uracil. Part of the cells were then harvested, washed, then suspended for 30 minutes at 37° in minimal salt solution in the absence of glycerol. The cells were harvested and suspended (4×10^8 cells per ml.) in 20 ml. of minimal salt solution; 2.0 μmoles of α-C^{14}-glycerol were added and 0.04 ml. of incubation mixture was removed, plated on platinum dishes and counted in a gas flow Geiger-Müller counter. The cultures were incubated for 1 hour at 37° with rapid swirling; 1 μmole of unlabeled glycerol was added to each flask and incubated 1 hour further. The flask contents were then centrifuged, and carrier non-labeled US, DHO, and OA (with mutant 550-460) were dissolved in 6.0 ml. aliquots of supernatant solution. The solutions were acidified, concentrated *in vacuo* at 45° to about 1.0 ml., crystallized, and the solutions were decanted. The crystals were blotted with paper and similarly recrystallized twice from water. Further crystallization showed no changes in specific activity, except as indicated.

* Samples largely contaminated by C^{14}-orotic acid.

DISCUSSION

The results presented in this paper and the preceding one (8) indicate the means by which pyrimidine biosynthesis may be regulated in *E. coli*. The first observation was that the growth factors uracil and cytosine had inhibitory effects on the formation of ureidosuccinic acid (US) by pyrimidine-requiring *E. coli* mutants. Inhibition of production of dihydroorotic acid (DHO) and orotic acid (OA), later products of the pyrimidine biosynthetic pathway, appears to be only a secondary effect of the earlier metabolic block.

It was reasoned that the inhibition occurred in the formation of US itself, rather than in the formation of its precursors. Demonstration that cytidine and cytidine-5′-phosphate actually were competitive inhibitors of aspartic acid and carbamyl phosphate in the formation of US *in vitro* proved this point. The observed inhibition constants were approximately

the same as those estimated by experiments *in vivo*. The closeness of the agreement of results *in vivo* and *in vitro* is, however, fortuitous.

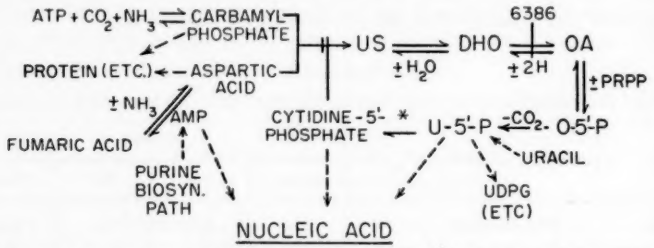

DIAGRAM 1.*, the actual conversion of the uridine derivative to the cytidine derivative was found to occur at the triphosphate level (13).

The last point of evidence was a demonstration of the operation of a regulatory mechanism in the cell. Normally, mutant 6386, which lacks the enzyme dihydroorotic dehydrogenase, can grow rapidly with OA as a growth factor and yet can produce large amounts of the pyrimidine intermediates US and DHO. However, when cells were irradiated with ultraviolet light to halt nucleic acid synthesis and placed in a medium containing OA, the production of the intermediates was quickly inhibited, although the production continued if the cells were in minimal medium. In these ultraviolet-irradiated cells, presumably, the cytidylic acid could not be removed by nucleic acid synthesis as it was being formed from OA, and it served to inhibit US formation as its concentration in the cell increased.

These results support the scheme shown in Diagram 1 for the self-regulated synthesis of pyrimidines by *E. coli*. The individual steps (solid arrows) have all been identified as enzymatic reaction (11, 13, 14, 8).

The operation of the scheme has already been indicated. If an external factor accelerated nucleic acid synthesis, the cytidylic acid concentration of the cell would decrease, thus allowing increased rate of pyrimidine synthesis. If nucleic acid synthesis were slowed or halted, the cytidylic acid (or derivative) concentration would rise and inhibit further pyrimidine biosynthesis. The location of the block is favorable to the cellular economy, since formation of US is essentially an irreversible reaction, while formation of both precursors (aspartate and carbamyl phosphate) involved freely reversible reactions. A block at a later point would thus be inefficient, since, as in the pyrimidine mutants, US would be produced whether it were needed or not, thus wasting energy and metabolites.

A second controlling factor may operate in the system for formation of aspartic acid. Fumaric acid plus ammonia is reported to be converted to aspartic acid in *E. coli* by either aspartase I (15) or aspartase II (16),

the latter enzyme being the most active and being activated by adenylic acid or adenosine. Increase in adenylic acid formation could increase the rate of aspartic acid formation via aspartase II, thus stimulating protein and pyrimidine biosynthesis and thus increasing the rate of nucleic acid synthesis. This effect would complement the stimulation of pyrimidine biosynthesis by decrease in cytidylic acid concentration, for the latter effect merely allows increased US formation if enough aspartic acid and carbamyl phosphate are present.

It seems likely that the rates of many biosynthetic processes in the cell are controlled in a manner similar to that for pyrimidine biosynthesis, since there have been several observations in the literature of end-products blocking their own formation. Probably adenylic or guanylic acid will be found to inhibit the first step in purine biosynthesis that is not freely reversible, and some amino acids will be found similarly to regulate their biosyntheses. The fact that such control mechanisms are wide-spread was indicated here by the metabolism experiments. Gearing of all pathways to the rate of growth would be very important to cells growing slowly in medium deficient in some factor. If a few syntheses were to be uncontrolled, metabolites might be wasted, thus resulting in less cell growth.

These observations may be important for another reason, since it is probable that such normal self-control systems in the cell are the target of inhibitors which are analogues of the natural end-products of such systems. Since such inhibitors are effective, although only traces of the normal metabolite are able to permit growth, the inhibitor must act not by preventing use of the metabolite, but rather by preventing its formation. Knowledge of the exact natural inhibitor of various systems could aid in synthesis of more effective inhibitors. For example, cytidine analogues should, on a theoretical basis, be very effective inhibitors of nucleic acid synthesis (more so than uracil or cytosine derivatives, for example), and this has been found to be true (17).

SUMMARY

1. Low levels (10^{-5} M) of uracil or cytosine, but not of thymine, inhibit formation *in vivo* of the pyrimidine intermediate ureidosuccinic acid in two pyrimidine-requiring mutants of *Escherichia coli*. Formation of the later consecutive intermediates dihydroorotic acid and orotic acid was halted by the absence of ureidosuccinic acid in the cells.

2. The inhibition is not an effect of growth itself, since one mutant (6386) with orotic acid as a growth factor could grow and yet produce ureidosuccinic and dihydroorotic acids.

3. Mutant 6386, in which nucleic acid synthesis was halted by ultraviolet irradiation, produced dihydroorotic acid in the absence, but not in the presence, of orotic acid in the medium.

4. Experiments *in vivo* with the irradiated mutants indicated a concentration of roughly 5×10^{-3} M unbound pyrimidine in the cell necessary to cause half inhibition of US formation.

5. Experiments *in vitro* showed that cytidine and especially cytidine-5′-phosphate were competitive inhibitors with aspartic acid and carbamyl phosphate for ureidosuccinic acid formation. K_i for the cytidylic acid was about 6×10^{-3} M. Uracil, uridine, uridine-5′-phosphate, and cytosine did not inhibit appreciably.

6. Both mutants 6386 and 550-460 in the absence of growth factors synthesized the pyrimidine intermediates at the rate normal for pyrimidine synthesis by wild type cells, but could convert 50 to 55 per cent of the ammonia and 13 to 20 per cent of the glycerol carbon to the intermediates. The normal incorporation into the pyrimidines of growing cells is 4 per cent of the ammonia and 2 per cent of the carbon from the medium.

7. The above facts indicate the existence of a cellular mechanism which links pyrimidine production to the rate of pyrimidine uptake for nucleic acid synthesis. Inhibition by an end-product of its own synthesis appears to be a common control mechanism in the cell.

Addendum—Umbarger (18) has presented preliminary evidence for a feed-back mechanism in isoleucine biosynthesis.

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ARGININE-ORNITHINE TRANSAMIDINATION IN KIDNEY*

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An enzyme preparation from hog kidney has recently been found to catalyze the reversible transfer of an amidine group from canavanine to ornithine, with the formation of canaline and arginine (1). Inasmuch as canavanine can serve as a substrate for certain enzymes whose usual substrate is arginine, *e.g.* arginase (2, 3) and argininosuccinase (4, 5), a reaction mechanism was suggested which involves the reversible formation of the same relatively long lived enzyme-amidine complex from either canavanine or arginine (1). If this proposed mechanism is correct, the same enzyme preparation should catalyze an arginine-ornithine transamidination reaction. In this paper evidence for the occurrence of such a reaction, obtained by the employment of radioactive ornithine, will be presented.

EXPERIMENTAL

Materials—DL-Ornithine-2-C^{14} monohydrochloride, with a specific activity of 0.63 mc. per mmole, was purchased from Tracerlab, Inc. (lot No. 22-92). In the experiments to be described, this compound was used directly, without the addition of non-labeled ornithine. The enzyme preparation from hog kidney, employed to demonstrate arginine-ornithine transamidination, was identical with that used previously to demonstrate both canavanine-ornithine and canavanine-glycine transamidinations (1). This preparation consists of an acetone powder of a dialyzed, 30 to 40 per cent ammonium sulfate fraction from an extract of acetone-dried hog kidney (1). A similar preparation from a 20 to 30 per cent ammonium sulfate fraction was employed as a source of argininosuccinase, as described elsewhere (5, 6). At the start of an experiment, the desired quantity of acetone powder was transferred directly to the reaction mixture. All incubations were carried out in the presence of toluene to prevent bacterial contamination.

Methods—The time-courses of the reactions to be described were followed

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by means of paper chromatography, by use of the ascending procedure of Williams and Kirby (7). After considerable experimentation, a single solvent system was found which effectively resolved the three compounds of primary interest in this investigation. With this solvent system, composed of 80 volumes of 95 per cent ethanol, 20 volumes of water, and 10 volumes of concentrated ammonium hydroxide, argininosuccinic acid has an R_f of 0.17, arginine 0.38, and ornithine 0.53. In some instances solvent systems of water-saturated phenol, with an ammonia atmosphere, and *n*-butanol-acetic acid-water (4:1:1) were employed. Approximately 2 to 3 μl. of the reaction mixture under examination were applied to each spot. After the chromatograms were run and dried, they were lightly sprayed with a 0.2 per cent solution of ninhydrin in 95 per cent ethanol to locate the separated compounds. The selected areas were cut out, stapled to filter paper wicks, and eluted with distilled water directly onto 1 inch copper planchets; the solutions were then dried and counted with a thin window Geiger tube and scaler (background 40 c.p.m.). In the experiments summarized in Figs. 1 and 2, the amount of radioactivity applied to each spot ranged between 1000 and 1500 c.p.m., as measured under the above conditions; in the experiment shown in Fig. 3, the total measured radioactivity applied to each spot was approximately 350 c.p.m. above background. The calculations involved in determining the relative radioactivities in Figs. 1 and 2 were based on the assumption that only L-ornithine participates in the reaction.

Results

Mammalian kidney cannot synthesize arginine from ornithine by the conventional pathway; on the other hand, this tissue does possess weak arginase activity. Consequently, for minimal ambiguity in the interpretation of results, the demonstration of an arginine-ornithine transamidination reaction should preferably be carried out with ornithine, rather than arginine, as the initially labeled compound. The postulated transamidination reaction would in that case be detected experimentally as a net transfer of label from ornithine to arginine, as represented by Reaction 1.

(1) $$\text{Ornithine-2-C}^{14} + \text{arginine} \rightarrow \text{arginine-2-C}^{14} + \text{ornithine}$$

Preliminary experiments established that such a reaction does indeed occur. When all reaction components are present, radioactivity is incorporated into arginine, and the shapes of the arginine-ninhydrin spots on the different chromatograms are identical with those of the corresponding spots on the autoradiographs. However, when arginine was omitted from the incubation mixture, no radioactivity could be detected at the R_f positions normally occupied by arginine in each of the three solvent systems

employed, even when, as an added control, arginine was added to the spots before running the chromatograms. In Fig. 1 are shown the results of a typical experiment in which the time-course of the reaction was followed; each point represents an average of several determinations. It can be seen that, as the reaction progresses, the increase in the radioactivity of arginine

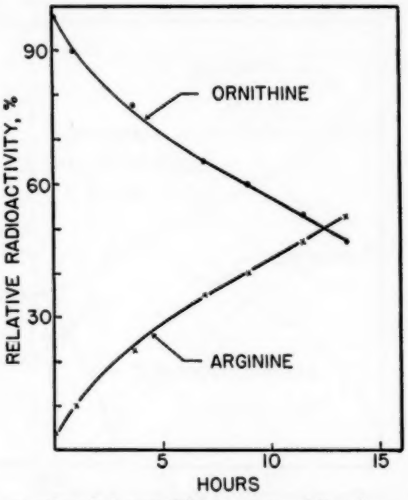

FIG. 1. Time-course of arginine-ornithine transamidination. A reaction mixture composed of 2 mg. of DL-ornithine-2-C^{14} monohydrochloride, 14 mg. of L-arginine monohydrochloride, and 5 mg. of kidney transamidinase in 0.9 ml. of 0.05 M potassium phosphate buffer, pH 7.2, was incubated at 35°. At the times indicated, aliquots were withdrawn for paper chromatographic analysis, with the use of an ethanol-water-ammonia solvent system. The formation of radioactive arginine at the expense of the active optical isomer of ornithine can be clearly seen.

is accompanied by a corresponding decrease in the radioactivity of ornithine, as would be expected if the enzyme were catalyzing Reaction 1.

The identification of the new radioactive reaction product as arginine was further confirmed by its enzymatic conversion to radioactive argininosuccinic acid (8, 9) after incubation with kidney argininosuccinase (5, 6) and fumarate. The results of a typical experiment are shown in Fig. 2. In this experiment labeled ornithine was incubated as before with non-labeled arginine in the presence of transamidinase for 24 hours; as in Fig. 1, radioactivity was incorporated into arginine at the expense of ornithine. At the end of this period, argininosuccinase and fumarate were added to the reaction mixture. It can be seen that the newly formed radioactive argi-

nine was largely converted to radioactive argininosuccinate, in accordance with Reaction 2. The shape of the argininosuccinate-ninhydrin spot corresponded exactly with that of the spot formed on exposure to x-ray film. When arginine was omitted from the reaction mixture, autoradiographs showed no radioactive spots other than that from the initially labeled ornithine.

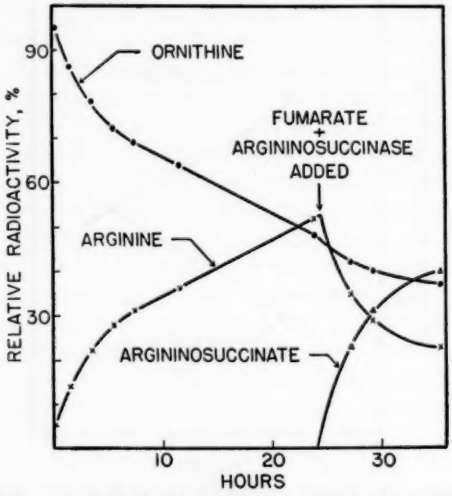

FIG. 2. Identification of radioactive arginine as a reaction product by its enzymatic conversion to radioactive argininosuccinate. A reaction mixture of 2 mg. of DL-ornithine-2-C^{14} monohydrochloride, 4 mg. of L-arginine monohydrochloride, and 8 mg. of kidney transamidinase in 0.9 ml. of 0.05 M potassium phosphate buffer, pH 7.2, was incubated for 24 hours at room temperature. At the time indicated by the arrow, 7 mg. of kidney argininosuccinase and 0.2 ml. of 0.8 M potassium fumarate were added to the reaction mixture. The subsequent formation of radioactive argininosuccinate at the expense of labeled arginine is clearly shown, as revealed by paper chromatographic analysis with an ethanol-water-ammonia solvent system.

(2) Arginine-2-C^{14} + fumarate $\rightleftarrows$ argininosuccinate-2-C^{14}

The radioactive argininosuccinate formed was further identified as follows. The deproteinized supernatant solutions resulting from several such experiments were combined, and radioactive argininosuccinate was isolated by elution from paper chromatograms and removal of water *in vacuo;* the isolated compound was free from radioactive impurities. After incubation of an aliquot with argininosuccinase, a 26 per cent reconversion of the isolated radioactive argininosuccinate into radioactive arginine was obtained, as would be predicted by a partial reversal of Reaction 2. Another ali-

quot of the radioactive argininosuccinate was heated at 100° with 0.5 N potassium hydroxide, and the time-course of the hydrolysis was followed for several hours (Fig. 3). As the ninhydrin spot corresponding to argininosuccinate disappeared, new spots corresponding to ornithine and aspartate appeared, thus completing the identification of argininosuccinate (8). As expected, the radioactivity from the argininosuccinate which was hydrolyzed appeared in the ornithine formed by the alkaline hydrolysis (Fig. 3).

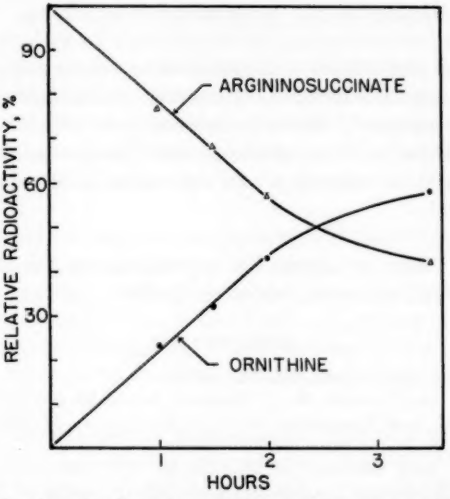

FIG. 3. Identification of radioactive argininosuccinate as a product of the reaction shown in Fig. 2 and localization of the label in the ornithine moiety by means of alkaline hydrolysis. Radioactive argininosuccinate, isolated by paper chromatography, was heated with 0.5 N potassium hydroxide at 100°, and the hydrolysis was followed by paper chromatographic analyses at the times indicated, with water-saturated phenol as solvent, under an ammonia atmosphere. Argininosuccinate was identified by the formation of ornithine and aspartate; the radioactivity was found to reside in the ornithine moiety.

DISCUSSION

The results of this radioactive exchange study and the canavanine-ornithine transamidination reaction can both be explained by assuming the reversible formation of a relatively long lived enzyme-amidine intermediate,

$$\text{Arginine} + \text{enzyme} \rightleftarrows \text{ornithine} + \text{enzyme}\text{—}\overset{\overset{\displaystyle NH_2^+}{\|}}{C}\text{—}NH_2 \qquad (3)$$

as suggested previously (1), and formulated as Reaction 3. The nature of

the linkage of the amidine group to the enzyme is at present unknown; it is possible that the amidine carbon may be linked to oxygen as in *O*-methylisourea, to nitrogen as in arginine, or, more likely, to sulfur as in *S*-methylisothiourea. In view of the importance of guanidino compounds in metabolism, this study will be continued.

SUMMARY

An enzyme preparation from hog kidney was found to catalyze a reversible transfer of the amidine group of arginine to ornithine. The radioactive arginine, formed by incubating non-labeled arginine with DL-ornithine-2-C^{14} and the enzyme preparation, was identified by converting it enzymatically to radioactive argininosuccinate. Inasmuch as the same enzyme preparation had been previously shown to catalyze a reversible canavanine-ornithine transamidination, it is postulated that a relatively long lived enzyme-amidine complex is formed as an intermediate in arginine-ornithine transamidination.

The author wishes to express his appreciation to Dr. R. H. Burris for his valuable contributions to this investigation.

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THE KINETICS OF IRON METABOLISM IN RAT LIVER SLICES

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In previous papers (1, 2) we have described the effect of various physical and chemical environments in the accumulation of iron by rat liver slices. It was shown by inhibition studies that metabolic energy is not directly linked to the accumulation of iron against an apparent concentration gradient. This is in contrast to the uptake of most other metal ions which are energy-dependent.

The mechanisms by which iron is transported through the mucosal walls as postulated by Granick (3) involve the participation of ferritin as the iron carrier. Alimentary iron seems to be prevented from entering the intestinal cells by a membrane mucosal block. The liver system does not appear to have such a mucosal block, as evidenced by the lack of stimulation of iron uptake when the liver cell membranes are ruptured by physical or chemical means (2).

By the application of kinetic studies to the liver slice system, we have been able to obtain a more intimate understanding of the processes involved. It will be shown that iron is sorbed on specific sites on an iron-binding entity at a rate limited by a diffusion-controlled transport of the iron.

Methods

Our procedures were essentially those described previously (1). For any one experiment, slices were obtained from the same liver. This was found to be necessary because of the variability among different livers. By such a procedure, internal standardization is achieved.

The slices are washed with cold Krebs-Ringer phosphate buffer (4), then placed in 3.0 ml. of Krebs-Ringer phosphate buffer, pH 7.0, containing 30 γ per ml. of iron as ferric ammonium citrate. It is important that these manipulations be carried out quickly and in the cold. The slices in beakers are incubated in Dubnoff metabolic shaking incubators and permitted to accumulate iron at 37° and 3° for various periods of time. The tissues are then removed, washed, and digested with 2 ml. of H_2SO_4:HNO_3 (1:1 of the concentrated acids) under a reflux. Total iron is determined colorimetrically by a modified thiocyanate procedure (5).

RESULTS AND DISCUSSION

The data from a typical experiment are presented in Fig. 1. Two important characteristics of these curves are (1) that the rate of accumulation is enhanced at the higher temperature and (2) that the capacity of the iron-binding entity to accumulate iron is increased at the higher temperatures.

We postulate that the process of iron accumulation by liver slices consists of two steps: (1) a diffusion-controlled transport to the sites on the iron-binding entity which is capable of being saturated, and (2) subsequent binding of the iron by a reaction or series of reactions, the rate of which is

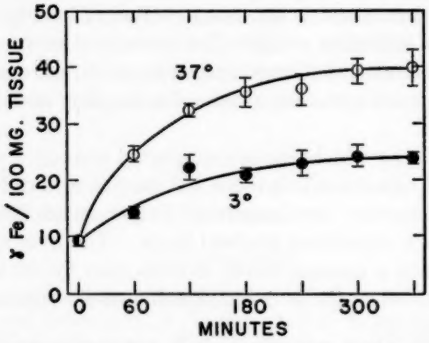

FIG. 1. Accumulation of iron by rat liver slices at 3° and 37° as a function of time. Rat liver slices were incubated in 3.0 ml. of Krebs-Ringer phosphate buffer, pH 7.0, containing 30 γ of iron per ml. as ferric ammonium citrate in a Dubnoff shaking incubator under an atmosphere of air at 3°(●) and 37°(○). At various time intervals the slices were removed, washed, and digested in HNO_3-H_2SO_4. Total iron concentration was measured by the thiocyanate procedure.

rapid compared to the rate of transport. On the basis of these assumptions, the rate of accumulation should be proportional to the number of sites on the iron-binding entity which are not occupied. Since the total number of sites should be proportional to the total amount of iron sorbed after an infinite time, and the number of sites occupied at any time t should be proportional to the total amount of iron sorbed up to time t, we can write for the rate of uptake the following equation:

$$\frac{-dQ}{dt} = k(Q_\infty - Q) \tag{1}$$

where Q = the amount of iron at time t, Q_∞ = the amount of iron after infinite time, and k = the rate constant.

The rate constant in Equation 1 should, if our assumption of diffusion

control of transport is correct, be directly proportional to the diffusion coefficient of the iron in whatever form it may be diffusing. It would be expected, therefore, that the activation energy A, obtained from the Arrhenius equation

$$\ln \frac{k_2}{k_1} = \frac{A}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \tag{2}$$

would be small, in general less than 10 kilocalories.

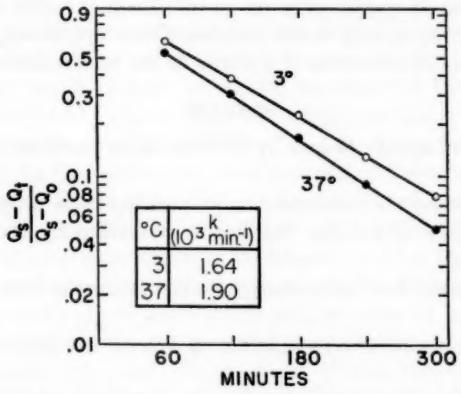

FIG. 2. Determination of rate constants for the uptake of iron by rat liver slices. The data from Fig. 1 are shown plotted as $\log((Q_s - Q)/(Q_s - Q_0))$ *versus* t. ○ represents 3°; ●, 37°. The slopes of the straight lines are equal to 2.3 k, where k is equal to the rate constant. The insert indicates the values for k so obtained. The apparent activation energy is 770 calories.

Upon integration of Equation 1, we find that

$$\ln \left(\frac{Q_s - Q}{Q_s - Q_0}\right) = -kt \tag{3}$$

where Q_0 = the amount of iron initially present.

On plotting the data from Fig. 1 as $\log (Q_s - Q)/(Q_s - Q_0)$ *versus* t we find, as postulated, the straight lines shown in Fig. 2 whose slopes are equal to 2.3 k. The insert in Fig. 2 shows the values obtained. The apparent A obtained from the rate constants is 770 calories, the low value of the apparent A being consistent with our kinetic interpretation.

The rate law Equation 3 indicates that the rate-determining kinetics of the iron uptake is of the first order. At present we have no definitive evidence of the nature of the ionic or non-ionic form of the iron transported.

From the fact[1] that ascorbic acid does not in any way influence the rate of accumulation of iron by rat liver slices, it does not appear that the ferrous form of iron is mandatory as postulated for intestinal transport (6). Our present investigation did not elucidate the mechanism of the non-rate-determining reaction or reactions at the active site on the iron-binding entity.

In conclusion, we believe that the accumulation of iron by rat liver slices against an apparent concentration gradient is not directly dependent upon metabolic energy. The metabolism consists of the specific uptake of the iron by sorption at specific sites on an iron-binding entity within the cell. The rate-determining step in the kinetics of iron uptake appears to be the diffusion-controlled transport of the iron to the unoccupied sites.

SUMMARY

1. The rate of uptake of iron by rat liver slices has been measured at 3° and 37°.

2. A rate law for the accumulation process has been proposed.

3. The validity of the rate law has been verified by the experimental results obtained.

4. The apparent activation energy for the process of iron uptake is 770 calories.

5. A mechanism for the metabolism of iron by rat liver slices has been proposed.

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METABOLISM OF C^{14}-LABELED ISONIAZID IN VITAMIN B_6-DEFICIENT RATS*

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(Received for publication, November 29, 1955)

Evidence indicating a relationship between isoniazid and vitamin B_6 has been accumulated recently. A syndrome resembling vitamin B_6 deficiency has been noted in man during the treatment of tuberculosis with high doses of isoniazid. These signs and symptoms have been alleviated by the administration of pyridoxine (1, 2). Antagonism of isoniazid and pyridoxine and its derivatives has been reported in bacterial enzyme systems and in growing cultures (3–7). The toxicity of isoniazid was enhanced in vitamin B_6-deficient rats (8, 9). Epileptiform seizures, occasionally seen in long term vitamin B_6 deficiency, were readily produced by the administration of isoniazid to vitamin B_6-deficient animals.

The present study of the distribution and excretion of isoniazid and its metabolites by vitamin B_6-deficient and normal rats was undertaken to determine possible differences which might explain the toxic manifestations brought on by the drug, especially the increased toxicity in vitamin B_6-deficient animals.

Although the results of the study indicated a decreased rate of metabolism of the drug in the vitamin B_6-deficient rats, this alone could not explain the early convulsions and increased toxicity of the drug in the presence of the vitamin deficiency.

Methods

A group of 6 week-old Sprague-Dawley rats (average weight 130 gm.) received a vitamin B_6-deficient diet plus deoxypyridoxine, as described by Beaton *et al.* (10). The animals exhibited a profound deficiency in 30 days. Control animals of similar ages and weights received the same basal diet supplemented with daily intraperitoneal injections of 50 γ of pyridoxine. Male rats were used for the tissue distribution studies, whereas female animals were used for the detailed blood and urine studies.

Isoniazid (labeled with C^{14} in the carbonyl group, specific activity of 4.04 mc. per mmole), prepared by the method of Murray and Langham

* This work was carried out under the auspices of the Atomic Energy Commission.

(11), was used in this study. A saline solution of the labeled isoniazid was diluted with the non-labeled drug to give a final concentration of 4 mg. per ml. and a specific activity of 5.23×10^7 c.p.m.

A dose equivalent to 10 mg. per kilo of body weight was given to the animals used for distribution studies. The material was injected subcutaneously in the back of the neck. The rats were placed in individual glass metabolism cages for collection of urine, feces, and expired CO_2 (12). They were sacrificed by exsanguination at 1, 6, and 24 hours after injection. For detailed blood and urine studies, the material was injected intravenously into the external jugular bulb. In the latter experiments, urine samples were obtained by catheterization under ether anesthesia, and blood samples were obtained by incision of the tail vein. Assays of the blood and urine samples were made by direct plating, as were the tissue and fecal samples after they were homogenized (13). Bone samples were oxidized by the Van Slyke-Folch method (14) and counted as $BaCO_3$. All samples were counted in a windowless gas flow proportional counter (geometrical factor, 50 per cent). Standard self-absorption corrections were made on all samples.

Filter paper chromatography was used to separate the radioactive metabolites in the plasma and in the urine (15–17). The solvents used were 80 per cent *n*-propanol (4:1 volume per volume) and *n*-butanol saturated with water. To obtain plasma concentrations of sufficient activity for the radioautographs, a higher dose (15 mg. per kilo) was given intravenously and the kidneys were tied off to effect accumulation of metabolites in the blood. The chromatographic strips were developed in a manner similar to methods described previously (13, 17).

Results

Total recoveries of the injected C^{14} activity from tissue and excreta ranged from 90 to 109 per cent. A negligible amount of the C^{14} activity was given off as expired CO_2. Approximately 40 per cent of the recovered activity was present in the urine at the end of 1 hour and 90 per cent at the end of 6 hours. Less than 10 per cent of the administered C^{14} activity was excreted in the feces. At 1 hour after injection, the gastrointestinal tract contained 9.0 ± 3.5 per cent and the liver 3.4 ± 1.2 per cent of the recovered activity. The blood value at this time was 3.0 ± 2.0 per cent and the kidneys, lung, brain, and skin values were 2.1 ± 1.0, 0.6 ± 0.2, 0.4 ± 0.21, and 0.2 ± 0.18 per cent, respectively.

The data in Table I show the concentration of C^{14} activity in the various tissues as a function of time after subcutaneous administration of labeled isoniazid to vitamin B_6-deficient and control animals. The values are re-

ported in microgram equivalents of injected dose[1] to facilitate comparison of the results with those reported by Roth and Manthei (17), who studied the metabolism of C^{14}-isoniazid in normal mice.

TABLE I

Distribution of C^{14} Activity Following Subcutaneous Administration of C^{14}-Isoniazid to Control and Vitamin B_6-Deficient Rats

The results are expressed as microgram equivalents per gm. of tissue.

Tissue or sample	1 hr.		6 hrs.		24 hrs.	
	Controls*	Vitamin B_6-deficient†	Controls‡	Vitamin B_6-deficient§	Controls‖	Vitamin B_6-deficient¶
Brain	3.7	2.9	0.75	0.52**	0	0
Skin	5.6	5.7	0.31	0.69	0	0
Muscle	6.2	5.0	0.27	0.33	0	0
Bone (whole)	1.1	1.3	0.14	0.16	0	0
Liver	8.4	6.7	0.58	0.41	0.05	0.05
Spleen	7.4	6.9	1.38	0.37	0	0.05
Kidneys	19.9	19.2	1.27	0.73	0.02	0.22
Adrenals	7.8	4.9**	0.53	1.19	0	0
Lungs	8.3	6.5**	0.52	0.40	0.04	0.01
Heart	10.2	5.5**	0.37	0.29	0	0
Gastrointestinal tract and contents	11.4	10.0	7.56	4.05	0.48	0.28
Remains	5.0	5.6	0.50	0.94	0.29	0.20
Blood (whole)	7.6	5.7**	0.37	0.26**	0	0
Feces (range)	2.9–35.4	8.3–80.8	1.46–2.64	0.61–4.2	1.3	1.1
Fat (range)	0.9–2.55	1.0–5.2	0.07	0.18	0	0

* Average of four animals.
† Average of five animals.
‡ Average of three animals.
§ Average of six animals.
‖ One animal.
¶ Average of two animals.
** Noted values are significantly different from control values, as determined by "t"-testing at the 95 per cent confidence level.

At 1 hour after *subcutaneous* injection of C^{14}-isoniazid, the concentration of radioactivity was significantly *lower* in several of the tissues (*i.e.* the adrenals, lungs, heart, and blood) of vitamin B_6-deficient rats than in that of non-deficient control rats. At 6 hours, only the brain and blood concentrations were significantly lower in the animals on the deficient diet.

[1] The tissue radioactivity equivalent to the activity of 1 γ of the injected isoniazid.

The concentrations of C^{14} activity in the blood of normal and vitamin B_6-deficient rats after *intravenous* administration of labeled isoniazid are

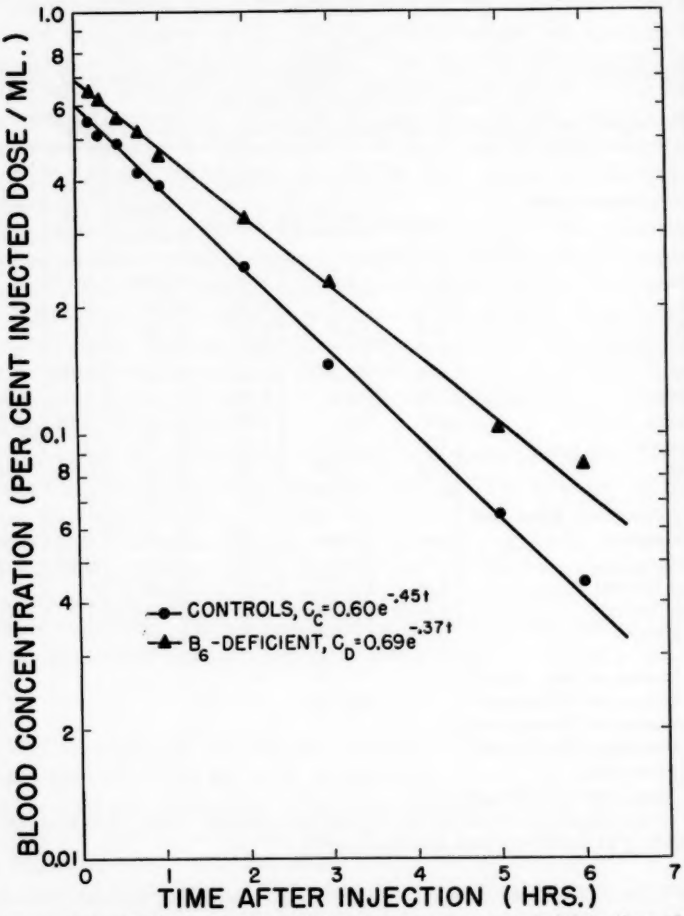

FIG. 1. C^{14} activity in the blood following intravenous administration of C^{14}-isoniazid in normal and vitamin B_6-deficient rats. Each point represents the average of three rats.

presented in Fig. 1, in which the log of the per cent injected dose per ml. of blood is plotted against time after injection in hours. When these data were analyzed by standard statistical methods, the slopes of the two regression lines were found to be significantly different, which showed that

the turnover half time of the C^{14} activity in the blood of the vitamin B_6-deficient animals was slower than that of animals on a normal diet. The turnover half times of the activity in blood following injection were 1.54 hours and 1.85 hours for the control and for the vitamin B_6-deficient animals, respectively. As expected, the zero time intercepts of the two curves were not significantly different.

The data on the cumulative urinary excretions of C^{14} activity, following intravenous administration of C^{14}-isoniazid to control and vitamin B_6-deficient animals, are given in Fig. 2. After intravenous injection, the blood values were consistently *higher* in the vitamin B_6-deficient animals, while the values for cumulative urinary excretion were lower.

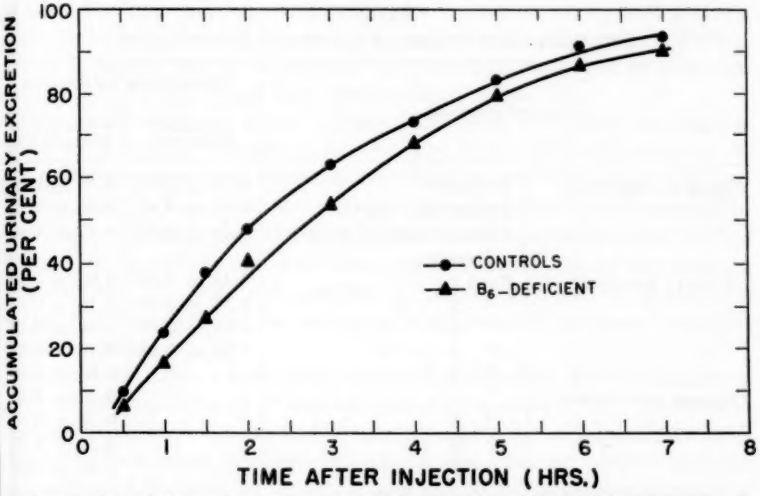

FIG. 2. Cumulative urinary excretion of C^{14} activity following intravenous administration of C^{14}-isoniazid in normal and vitamin B_6-deficient rats. Each point represents the average of three rats.

Chromatograms of plasma (developed with the *n*-butanol-water system) 1 hour after injection of C^{14}-isoniazid gave three definite bands in both the control and vitamin B_6-deficient rats. At 6 hours after injection of the drug, chromatograms of plasma from control animals showed the presence of four metabolites, while those of plasma from the vitamin B_6-deficient animals exhibited only three distinct bands. At 12 hours, chromatograms of the plasma from most of the animals in both groups showed the presence of four metabolites and, at 24 hours after injection, the chromatograms of the urines of all animals showed four metabolites. The R_f values of the

metabolites in plasma (with the *n*-butanol-water system) are given in Table II. Evidence of a fifth band was seen on some chromatographic strips developed with 80 per cent *n*-propanol.

Although there were no differences in the final number of metabolites in the urines of control and vitamin B_6-deficient rats, chromatographic studies indicated variations in their rate of formation or their time of appearance in the plasma and urine. At 6 hours after intravenous injection of the isoniazid, only two of the four urinary metabolites present at earlier times were

TABLE II

R_F Values for C^{14}-Isoniazid and Some of Its Metabolites

Compound or material		Solvent system and R_F values	
		n-Propanol 80 per cent	*n*-Butanol saturated with H_2O
Known compounds	Isoniazid*	0.66 ± 0.04	0.43 ± 0.04
	Isonicotinic acid*	0.41 ± 0.03	0.16 ± 0.03
	1-Isonicotinoyl-2-acetylhydrazine†	0.76 ± 0.04	0.63 ± 0.03
Urinary metabolites‡	Band 1	0.40 ± 0.02	0.05 ± 0.02
	" 2	0.54 ± 0.03	0.15 ± 0.04
	" 3	0.64 ± 0.03	0.23 ± 0.02
	" 4	0.80 ± 0.03	0.40 ± 0.05
	" 5		0.63 ± 0.03
Plasma metabolites	" 1		0.10 ± 0.04
	" 2		0.23 ± 0.02
	" 3		0.38 ± 0.03
	" 4		0.68 ± 0.04

* Determined with C^{14}-labeled compounds.

† Determined with the CNBr-benzidine color test (18).

‡ All the values are the average of eight samples, four controls, and four vitamin B_6-deficient rats.

still present in the urine of the control animals, while at least three to four were still detectable in the urines of the vitamin B_6-deficient rats. The bands with R_F values of 0.54 and 0.8 (with 80 per cent *n*-propanol) were most consistently present in the urine of control animals 6 hours after injection of the drug. Bands with R_F values of 0.4, 0.54, 0.8, and occasional traces of a material with an R_F value of 0.64 predominated in urine of the vitamin B_6-deficient animals.

Table II indicates the urinary metabolites (and their R_F values) that appeared consistently in all animals. When the 80 per cent propanol solvent was used, some metabolites appeared inconsistently; these had R_F values of 0.23 and 0.92. For purposes of comparison, the R_F values

of several related known compounds also are given. A comparison of the R_F values found in the urine with those for the known compounds, isoniazid, isonicotinic acid, and 1-isonicotinoyl-2-acetylhydrazine serves to identify these compounds as metabolites of isoniazid. This result is in agreement with previously reported observations (16, 17, 19, 20).

DISCUSSION

Tissue concentrations and excretion rates of the drug and its metabolites were similar to those reported for the mouse (17), with two exceptions. In the rat, the lungs did not contain significantly greater amounts of activity than the blood. The activity was not localized in the skin and it was eliminated so rapidly that it is doubtful whether the skin served as a storage depot for the drug, as had been postulated by others (17).

The gastrointestinal tract, kidneys, and liver contained the highest amounts of activity per gm. of tissue. The high tissue concentration in the gastrointestinal tract suggested that the drug may undergo metabolism in this organ. There was also evidence for metabolism of the material in the liver. Not only were the drug and its derivatives concentrated in this organ, but in addition an acetylated derivative was one of the primary metabolites. The high concentration in the kidneys was, no doubt, due to the fact that this was the major excretory route of unchanged isoniazid and its metabolites.

Plasma and urinary metabolites detected in this study were in agreement with those reported by Zamboni and Defranceschi (19). Unchanged isoniazid, isonicotinic acid, and the acetyl derivative of isoniazid are well established excretory products (16, 17, 19, 20). The metabolite with the R_F value of 0.54 (with 80 per cent *n*-propanol as the solvent) corresponded closely to the R_F value of 0.50 obtained for pyruvic acid isonicotinoyl hydrazine, with 85 per cent isopropanol as the solvent (19).

No evidence for the formation of pyridoxal isonicotinoyl hydrazone (postulated by Biehl *et al.* (1)) was noted in this study. Control chromatograms with a known sample showed that the above compound could be detected as a bright fluorescent band (R_F value of 0.67 $\pm$ 0.04) with *n*-butanol saturated with water as the solvent. No such fluorescent band was present as a urinary metabolite. It is possible that hydrolysis of the compound could have occurred in the urine prior to or during the process of chromatography.

From isotope dilution calculations (21) the dilution volume of isoniazid and its metabolites was 64 to 74 per cent of the body weight. Since this approximated body water, in all probability these materials not only pass from the blood into extracellular fluid but also into intracellular water.

The most significant finding in the present study was the lower rate of

metabolism of the drug in the vitamin B_6-deficient rats when compared with that of the animals on a normal diet. The lower rate of metabolism was manifested in several ways. After intravenous injection of the labeled isoniazid, the appearance of C^{14} activity in the urine of the vitamin B_6-deficient rats was significantly delayed. The delay was mirrored by higher radioactivity values in the blood and a 20 per cent longer turnover half time. On the other hand, after *subcutaneous* injection of the drug, the blood values were lower in the vitamin B_6-deficient animals. One might explain this apparent reversal of results by the decreased rate of absorption from the injection site, again an indication of decreased metabolism. It is also of interest that the highly vascular organs, such as the heart and lungs, of the vitamin B_6-deficient animals showed lower tissue concentration values after subcutaneous injection than did those of the control animals. The lower concentration in the soft tissue may be explained on the basis of the lower blood values in the latter group of animals. There is no readily apparent explanation of the significantly higher tissue concentrations in the adrenals of the control group over the vitamin B_6-deficient animals.

The lower rate of metabolism is also observed in the chromatographic studies of plasma and urine, which suggested a much slower appearance of the metabolites in the fluids of the vitamin B_6-deficient animals. At 6 hours, the control animals showed four metabolites in the plasma, while those on the deficient diet had only three. This was also reflected in the urinary metabolites with the vitamin B_6-deficient animals excreting some of the metabolites over a longer period of time.

The differences in the metabolism of C^{14}-isoniazid in the vitamin B_6-deficient rats could be explained on the basis of the general decreased rate of metabolism associated with vitamin deficiency. These differences may only partially explain the increased toxicity of isoniazid in vitamin B_6-deficient rats.

SUMMARY

C^{14}-labeled isoniazid was administered to normal and vitamin B_6-deficient rats. 90 per cent of the C^{14} activity, as changed or unchanged drug, was excreted in the urine 6 hours after injection of isoniazid. At 1 hour after subcutaneous injection of the drug, tissue concentrations of C^{14} activity as isoniazid or its metabolites were significantly lower in the blood, lungs, heart, and adrenals of the vitamin B_6-deficient rats. At 6 hours after injection, only the blood and brain of the vitamin B_6-deficient animals had significantly lower concentration of activity than the controls. The turnover half time of isoniazid and its metabolites in the blood was 1.54 hours in the control animals and 1.85 hours in the animals on the deficient diet. The rate of metabolism and excretion of isoniazid and its metabolites was

decreased in the vitamin B_6-deficient rats. No complete explanation for the increased toxicity of isoniazid in vitamin B_6-deficient rats could be found in this study.

The authors wish to acknowledge the advice of Dr. Harry Foreman. We are indebted to Arthur Murray, III, for the synthesis of the pyridoxal isonicotinoyl hydrazone and to Dr. Donald G. Ott for the preparation of 1-isonicotinoyl-2-acetylhydrazine.

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POLYSACCHARIDE FORMATION IN REPAIR TISSUE DURING ASCORBIC ACID DEFICIENCY*

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Formation of a normal fibrous repair tissue requires ascorbic acid, for in the absence of this vitamin a tissue proliferates lacking the fibrillar elements which contribute to tensile strength (1). Chemical analyses have shown that the massive repair tissue induced by the subcutaneous injection of a foreign body, the galactan carrageenan, contained very little collagen when the animals received a diet deficient in ascorbic acid (2). This tissue had a higher water content than repair tissue from adequately fed guinea pigs and, when minced, exuded appreciable amounts of a viscid, mucinous fluid. A preliminary comparison of this fluid with the scanty serous exudate which appeared when normal repair tissue was minced showed that the former contained greater amounts of mucopolysaccharides.

The increased interest in connective tissue polysaccharides during recent years has prompted several investigations of the changes in these compounds during scurvy. Penney and Balfour (3) concluded, on the basis of metachromatic staining reactions, that acid mucopolysaccharides did not appear in muscle wounds of scorbutic guinea pigs, whereas Bunting and White (4), using similar techniques, reported that mucopolysaccharides were more abundant than normal in skin wounds of scorbutic guinea pigs. Persson (5) found that sections of various scorbutic tissues contained more hexosamine and yielded more intensive polysaccharide staining than the comparable normal tissues. Gersh and Catchpole (6) interpreted their observations as indicating a depolymerization of glycoproteins during scurvy, but did not consider the effect of scurvy on concentration. Ludwig (7) stimulated new connective tissue formation in normal and scorbutic guinea pigs by inducing exophthalmos. The retrobulbar tissue increased in both groups and there was no difference in hexosamine content. Kodicek and Loewi (8), in a recent publication, reported that the hexosamine content of repair tissue from tendons of normal and scorbutic guinea pigs was similar. Dunphy and Udupa (9) have referred to the increased mucopoly-

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saccharide content of scorbutic granulation tissue. All in all, the cited reports permit no clear conclusion concerning the formation and abundance of mucopolysaccharides in connective tissue during ascorbic acid deficiency.

The data presented in this paper show that excessive amounts of mucopolysaccharides accumulate in repair tissue which forms in the absence of adequate ascorbic acid.

EXPERIMENTAL

Analytical Methods—Assay of aminosugar was based on the method of Schloss (10). Slight modifications were the substitution of isopropanol for ethanol and changes in the composition of the carbonate buffer so that tissue or polysaccharide hydrolysate could be used without prior neutralization. Hydrolysis was carried out for 4 hours in 4 N HCl at 100° in glass test-tubes sealed with Teflon-lined screw caps. The hydrolysate was simply diluted prior to assay, but, if the concentration was low, an aliquot was evaporated to dryness *in vacuo* over NaOH and then made to a convenient volume with 0.4 N HCl. Glucosamine hydrochloride was always subjected to the same procedure and unknowns were corrected for recoveries which averaged 98 per cent. Nitrogen was determined by a micro-Kjeldahl method (distillation) and uronic acid by the carbazole method of Dische (11). Estimates of viscosity were made of polysaccharide dissolved in phosphate buffer[1] by using a Cannon-Fenske pipette with an efflux time for water of 60 seconds at 25°.

Preparation of Tissue—Proliferation of a repair tissue was induced in guinea pigs by subcutaneous injection of an extract of Irish moss,[2] previously described techniques being used (2). The Macdonald No. 5 scorbutigenic diet (12) was supplemented with 50 mg. of ascorbic acid every other day, but this supplement was discontinued after the Irish moss injection when a "scorbutic" repair tissue was desired. Repair tissue was removed from the guinea pigs on the 14th day following injection. Normal repair tissue was also taken at 8 days, before appreciable collagen had formed (2), in order to compare the polysaccharide content of this early stage tissue with that of the "scorbutic" repair tissue. The tissue, freed grossly of necrotic material, muscle, and blood, was minced with scissors and then forced through a tissue press with a plate having 0.8 mm. holes.

Analysis of numerous normal and ascorbic acid-deficient repair tissues

[1] The phosphate-sodium chloride buffer used throughout this study had a pH = 7.0 and a $\Gamma/2 = 0.26$.

[2] We are indebted to Mr. L. Stoloff, Seaplant Chemical Corporation, New Bedford, Massachusetts, for a purified preparation.

yielded the average values which are given in the accompanying table:

The values are given in mg. per gm. of fresh tissue.

	Water	Nitrogen	Collagen	Hexosamine
Normal tissue	868	16.8	15.3	1.6
"Scorbutic" tissue	895	14.5	2.4	1.8

As shown above, the "scorbutic" tissue contained significantly less collagen and more water than the normal tissue, but the total nitrogen content was about the same, especially if corrected to a dry weight basis. In view of the errors involved in the determination of hexosamine in proteinaceous mixtures, the differences observed between the two tissues could be considered no more than suggestive of a difference in mucopolysaccharide content.

Extractable Mucopolysaccharides—In order to obtain a more reliable assay of mucopolysaccharides, it was necessary to separate these from the bulk of tissue proteins. Three different procedures were applied. In the first method, the tissue mash was extracted with neutral phosphate buffer, in the second, extraction with 2 per cent phenol was followed by extraction with M potassium thiocyanate, and in the third, the tissue was digested with pepsin and trypsin.

A phosphate extract was prepared by mixing 10 gm. of tissue mash in about 20 ml. of buffer and allowing it to stand for 3 days at 4° with occasional stirring. This was repeated twice and the pooled extracts were made to a volume of 70 ml. 25 gm. of potassium acetate were added to a 50 ml. aliquot and, after centrifuging sharply and filtering through glass wool, polysaccharide was precipitated from the filtrate by pouring the mixture into 150 ml. of ethanol. The precipitate was washed with ethanol and ether, then dissolved in 10 ml. of phosphate buffer. The solutions were clarified by centrifugation for 1 hour at 14,000 × g. Preparations so obtained from ascorbic acid-deficient tissue had a significantly higher viscosity and hexosamine content than those from normal repair tissue (Table I).

Since the hexosamine content of the polysaccharide fraction obtained after phosphate extraction was considerably less than the total hexosamine content of the tissue, more effective solvents, phenol and potassium thiocyanate, were used. 10 gm. of tissue mash were extracted first with 20 ml. of 2 per cent phenol, then with two 20 ml. portions of 1 M KCNS. The extraction and subsequent preparation of the polysaccharide fraction followed the procedure described for phosphate. Upon precipitation with ethanol, extracts from scorbutic tissue yielded fibrous precipitates in contrast to the

flocculent or finely amorphous precipitates obtained from normal repair tissue. The amount of aminopolysaccharides isolated from the ascorbic acid-deficient tissue, as indicated by hexosamine concentration, was again greater than that from the normal tissue (Table I). The relatively high viscosity of the polysaccharide solutions from ascorbic acid-deficient animals and the fibrous nature of the precipitates are suggestive of a highly polymerized compound. Although the degree of polymerization *in vivo*

TABLE I

Comparison of Polysaccharide Fraction from Normal and "Scorbutic" Repair Tissue

The values are given in mg. per gm. of fresh tissue.

Extraction procedure	State of tissue	No. guinea pigs	η_{rel}	Hexosamine	Nitrogen	Uronic acid
Phosphate buffer	Normal after 14 days	10	1.34 ±0.06*	0.06 ±0.004	0.125 ±0.003	
	Scorbutic after 14 days	11	1.94 ±0.21	0.33 ±0.040	0.147 ±0.010	
	Normal after 8 days	7	1.10 ±0.01	0.07 ±0.004	0.135 ±0.008	
Phenol-thiocyanate	Normal after 14 days	19	2.10 ±0.20	0.14 ±0.015	0.406 ±0.029	0.17 ±0.027
	Scorbutic after 14 days	16	6.75 ±0.47	0.73 ±0.059	0.454 ±0.010	0.64 ±0.108
	Normal after 8 days	6	2.41 ±0.38	0.21 ±0.007	0.503 ±0.032	0.14 ±0.009
Enzymatic digest	Normal after 14 days	12	1.08 ±0.01	0.24 ±0.009	0.234 ±0.022	
	Scorbutic after 14 days	7	1.49 ±0.02	0.80 ±0.058	0.268 ±0.009	

* The data are presented as the mean ± its standard deviation.

may be different, these data do not support the view that ascorbic acid deficiency produces a depolymerized connective tissue matrix (6).

In order to circumvent the possibility that these results represented only a difference in extractability, 10 gm. of tissue were incubated with 5 mg. of crystalline pepsin and maintained at pH 2 and 38° for 18 hours. At this time, another 5 mg. of pepsin were added and the digestion was continued for 18 hours. The pH was adjusted to 8 with 2 N K_2CO_3 and 10 mg. of crystalline trypsin were added. After incubation for 20 hours the small amount of insoluble residue was centrifuged and the polysaccharides were precipitated from the supernatant solution by addition of 5 volumes of ethanol. The precipitate was dissolved in 15 ml. of phosphate buffer, 5 gm. of potassium acetate were added to a 10 ml. aliquot, and the mixture was

centrifuged. The supernatant solution was poured into 30 ml. of ethanol; the precipitate obtained was washed with ethanol and ether, then dissolved in 10 ml. of phosphate buffer. Analyses of the polysaccharide fractions obtained in this way confirm the results of the two extraction procedures that the mucopolysaccharide fraction from "scorbutic" repair tissue contains 3 to 6 times as much hexosamine as that from normal repair tissue.

Immature Repair Tissue—The data presented here raise the question whether the increased concentration of polysaccharide is simply a failure to remove the polysaccharides which form in the early stages of granulation (13), or whether the cellular production of polysaccharide is increased by a deficiency of ascorbic acid. The polysaccharide fraction extracted from normal young repair tissue (8 days) in which there has not yet been appreciable collagen formation (2) yielded analytical data, also presented in Table I, similar to those from normal tissue. These data suggest an increased activity of the scorbutic polysaccharide-producing cells.

DISCUSSION

The classic morphologic description of ascorbic acid deficiency has been that of an inability to lay down and maintain a normal intercellular matrix (1). Histologists, in focusing their attention on one component of this matrix, believed that ascorbic acid was necessary for the maintenance as well as formation of normal collagen (1, 14, 15). Biochemical studies confirmed the necessity of ascorbic acid for collagen formation (2), but showed that the vitamin was unnecessary for maintenance of collagen once it had formed (16). The finding that excess polysaccharides accumulate in scorbutic repair tissue may help to reconcile the two views regarding collagen maintenance and to clarify some of the changes observed in scurvy.

We believe that these divergent views arose because the definitions of collagen used by biochemists and histologists are operational and different. The biochemists use the term collagen to refer to a molecular entity defined by its chemical reactivity and characteristic amino acid composition, *viz.* relative insolubility in dilute alkali, hydrolyzability to gelatin in boiling water, and high hydroxyproline content. The histologists, on the other hand, refer to a microscopic entity which has particular staining properties and a characteristic appearance when observed with the light microscope. Collagen so defined includes the molecular entity of the biochemist but probably includes mucopolysaccharides and other components of the intercellular matrix.

In normal connective tissue the molecular collagen fibrils with associated water and polysaccharides are lined up in parallel to form the characteristic undulating fibers observed by the histologist. Excessive amounts of

hydrophilic polysaccharides accumulate during an ascorbic acid deficiency, as suggested by the present data and by previous studies (5). These would disperse the fibrils and the collagen might be invisible with the microscope or appear as reticulin or fragmented fibers even though the molecular collagen itself had undergone no change detectable by chemical techniques. The tissue containing the separated fibrils might then be expected to have less tensile strength, even though the concentration of molecular collagen had not decreased. The ease with which skin of scorbutic guinea pigs tears despite an increased collagen concentration (16) may be an example of such behavior. An observation common in the older literature of scurvy (17) that long healed wounds broke open anew when an individual became scorbutic might thus be satisfactorily explained. Breakdown of a scar would have been especially likely if it had healed improperly and was chronically cellular as a consequence of the low ascorbic acid content of the then usual diet.

SUMMARY

Proliferation of a granulomatous repair tissue was induced in guinea pigs by the subcutaneous injection of a carrageenan suspension. The collagen-poor tissue that formed in guinea pigs deprived of ascorbic acid during tissue development contained about 5 times as much mucopolysaccharide as repair tissue that formed in animals adequately supplied with ascorbic acid. The predominant polysaccharide which accumulates in scorbutic repair tissue has been isolated and identified as hyaluronic acid.)

Excessive polysaccharide formation in scorbutic tissue was considered to be a factor responsible for the degradative histologic changes observed in collagen during scurvy.

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THE METABOLISM OF VITAMIN E

I. THE ABSORPTION AND EXCRETION OF *d*-α-TOCOPHERYL-5-METHYL-C^{14}-SUCCINATE*

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Vitamin E is widely distributed throughout the tissues of animals and man (2–4), and its deficiency causes a variety of syndromes in laboratory and farm animals (5). Very little information, however, is available concerning either the metabolic fate or the biochemical rôle of this vitamin. We have, therefore, undertaken a study of the metabolism of α-tocopherol.

It has been reported that α-tocopherol and α-tocopherylquinone were not excreted in the urine of animals even after the administration of large doses of vitamin E (6, 7). Some excretion of absorbed tocopherol into the intestinal tract has been postulated (7, 8). In this report we shall present definitive evidence both for the presence of a metabolic product of α-tocopherol in the urine and for the excretion of α-tocopherol and its metabolic products from the blood stream into the intestinal tract.

Materials and Methods

d-α-Tocopheryl-5-methyl-C^{14}-succinate—The crystalline succinate ester of *d*-α-tocopherol labeled with C^{14} in the 5-methyl group was used in these studies.[1] The material had a specific activity of 0.147 μc. per mg.

β-D-Glucuronidase—Beef spleen and rat liver preparations of β-D-glucuronidase (9) were used.[2]

Administration and Collection—Young rabbits (1 to 2 kilos) maintained on Diet 11 of Goettsch and Pappenheimer (10) supplemented with *dl*-α-tocopherol were kept in metabolism cages which permitted separation and

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A preliminary report on this work was presented before the 44th meeting of the American Society for Pharmacology and Experimental Therapeutics at Atlantic City, April, 1954 (1).

[1] The authors wish to thank Dr. P. L. Harris of Distillation Products Industries for making available the labeled *d*-α-tocopheryl succinate.

[2] The authors are indebted to Dr. M. Levitz of New York University College of Medicine, and Dr. D. Dziewiatkowski of The Rockefeller Institute for Medical Research for generous quantities of β-*d*-glucuronidase preparations.

complete collections of all feces and urine eliminated during each 24 hour period. The *d*-α-tocopheryl-5-methyl-C^{14}-succinate in doses of 10 to 15 mg. (1.5 to 2 μc.) was administered by various routes. The material was dissolved in 1 ml. of sesame oil for oral and subcutaneous administration. For intravenous injection it was dissolved in 1 ml. of a mixture of 9 parts of 5 per cent aqueous triethanolamine and 1 part of ethanol. Massive doses were administered by mixing 200 mg. of unlabeled *d*-α-tocopheryl succinate with the tracer dose in 6 ml. of aqueous triethanolamine-ethanol.

Extraction of Radioactive Materials—Urines were acidified to pH 1 to 2 and extracted continuously with ether for 48 hours. About 90 per cent of the radioactivity was recovered in this manner. Longer extraction times did not result in an increased yield of C^{14}. Feces were dried in vacuum and ground to a fine powder. Continuous extraction with ethanol in a Soxhlet apparatus for 24 hours yielded 80 to 90 per cent of the radioactivity. The yields were not improved by increasing the time of extraction.

Methods of Counting—Whenever levels of activity permitted, samples to be counted were converted to barium carbonate by a modification (11) of the wet combustion method of Van Slyke and Folch (12) and counted at "infinite thickness" in stainless steel planchets in a Tracerlab windowless gas flow counter. The factor relating counts per minute of "infinitely thick" barium carbonate to microcuries was determined by counting as barium carbonate a sample of *d*-α-tocopheryl-5-methyl-C^{14}-succinate of known weight and specific activity.

When combustion was not feasible, small urine aliquots were dried slowly in tared planchets under an infra-red lamp and counted. A self-absorption curve for these samples was constructed by counting a known quantity of *d*-α-tocopheryl-5-methyl-C^{14}-succinate with various quantities of urine residues. Samples of dried feces were counted directly at "infinite thickness." Conversion of counts per minute at "infinite thickness" to microcuries was accomplished by counting fecal samples of sufficiently high specific activity both directly and as barium carbonate. Agreement between counts by direct plating and combustion was reasonably good (±10 per cent).

Hydrolysis of Urinary Excretion Product; Acid Hydrolysis—To the residue of an evaporated ether extract of a 24 hour specimen of urine 100 ml. of 3 N HCl were added, and the mixture was refluxed for 2 hours. On cooling to room temperature the hydrolysis mixture was extracted with petroleum ether for 24 hours.

Enzyme Hydrolysis—The residue of an evaporated ether extract of a 24 hour specimen of urine was dissolved in 40 ml. of water, the pH was adjusted to 4.5 with solid sodium bicarbonate, and 4 ml. of acetate buffer (pH 4.5) were added. The mixture was incubated at 37° for 5 days with β-D-glu-

curonidase in concentration of 400 Fishman units per ml. Penicillin and Streptomycin, in concentrations of 25 units and 25 γ per ml., respectively, were added to prevent bacterial growth. The incubation mixture was subsequently extracted with petroleum ether for 24 hours.

Ultraviolet Spectra—Ultraviolet spectra were determined in purified ethanol (distilled over potassium permanganate and potassium hydroxide) and "spectrograde" isooctane (Phillips Petroleum) in silica cells with a 1 cm. light path in a Beckman DU spectrophotometer.

Paper Chromatography—Two reverse phase paper chromatographic systems, developed for the separation of the tocopherols, were employed: the system of F. Brown (13) as modified by Eggitt and Ward (14) in which Whatman No. 1 paper is impregnated with light petroleum, B. P., Nujol, and the chromatogram is developed with 75 per cent ethanol, and the system of J. A. Brown (15) in which Whatman No. 1 paper is treated with silicone stop-cock grease and developed with appropriate mixtures of acetonitrile and water. Spots were identified by scanning under an ultraviolet lamp and, in the case of α-tocopherol, by spraying with a ferric chloride-dipyridyl reagent in glacial acetic acid (16).

Isotope Dilution Technique—Known quantities of unlabeled *d*-α-tocopherol or *d*-α-tocopheryl succinate were added to extracts of feces or urine, and isolated as the crystalline succinate ester. In instances when free α-tocopherol had been added, the succinate was prepared by refluxing the dry residue with an excess of succinic anhydride in a few ml. of pyridine for 3 hours. Ether was added to precipitate the excess anhydride, which was removed by filtration. Repeated washings with 5 per cent HCl removed the pyridine. Ether was removed by evaporation. Isolation of tocopheryl succinate was achieved in the following manner: the mixture was dissolved in 83 per cent ethanol and made slightly alkaline. Extraction with three portions of petroleum ether removed impurities. After acidification of the ethanol solution, the tocopheryl succinate was extracted into petroleum ether. The crude tocopheryl succinate was recrystallized to constant specific activity from isooctane or petroleum ether. The total radioactivity of the tocopheryl succinate (specific activity times theoretical yield) divided by the total radioactivity in the extract represents the fraction of isotopic material present in the extract as tocopherol or tocopheryl succinate.

Isolation of Fecal Metabolite—A crude fecal metabolite was isolated by a modification of the series of steps described by F. Brown (13). *p*-Acetylaminophenol (17) was found to be a better antioxidant than pyrogallol during the saponification. In the chromatographic step α-tocopherol was eluted from Florex XXS with benzene; however, ether-ethanol mixtures were necessary to elute the metabolite.

RESULTS AND DISCUSSION

Absorption and Excretion Studies—The oral administration of a sesame oil solution of *d*-α-tocopheryl-5-methyl-C^{14}-succinate led to a rapid elimination of radioactivity in the feces, as is shown in Fig. 1. Over 74 per cent of the administered dose appeared in the stool within 3 days after administration. More than 93 per cent of the fecal excretion product was shown by the isotope dilution technique to be either α-tocopherol or α-tocopheryl succinate. Only traces of radioactivity were found in the urine.

The subcutaneous administration of labeled α-tocopheryl succinate in oil was followed by very slow elimination of radioactivity (Fig. 2). During

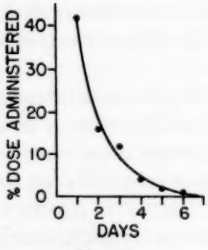

FIG. 1

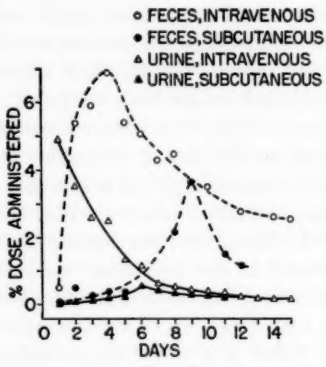

FIG. 2

FIG. 1. Excretion of radioactivity in feces after oral administration of a tracer dose of *d*-α-tocopheryl-5-methyl-C^{14}-succinate.

FIG. 2. Excretion patterns after subcutaneous and intravenous administration of tracer doses of *d*-α-tocopheryl-5-methyl-C^{14}-succinate.

11 days only 10 per cent of the dose appeared in the feces and 4 per cent in the urine. When the animal was sacrificed on the 11th day after injection, 14 per cent of the administered dose was found at the site of injection.

When the isotopic vitamin E was given by the intravenous route, considerable radioactivity was eliminated in both urine and feces, as shown in Fig. 2. The urinary activity was highest on the 1st day and decreased rapidly thereafter, while the fecal output reached its peak on the 3rd or 4th day after injection and fell off more slowly. A period of 15 to 20 days was required for complete elimination of the isotopic material as shown by the absence of detectable radioactivity from both urine and feces after this period. The total recovery of C^{14} from the excreta comprised 70 to 75 per cent of the administered dose. About 20 to 30 per cent of the recovered radioactivity appeared in the urine, while 70 to 80 per cent was found in the feces.

When a "massive dose" of tocopheryl succinate was administered by the intravenous route, the total amounts of radioactivity appearing daily in the excreta were roughly the same as when only the tracer dose was given. This would seem to indicate that the fraction of the dose that is eliminated by the body is essentially independent of the size of the dose in the range of 10 to 220 mg.

Isotope dilution studies showed that in the feces of rabbits given α-tocopheryl succinate either subcutaneously or intravenously 40 to 50 per cent of the ethanol-extractable excretion products was free α-tocopherol. Virtually all of the succinate ester had been hydrolyzed in the body.

Previous reports on the absorption of vitamin E are few and are limited largely to studies of absorption from the gastrointestinal tract (18, 19). The results were usually based on the chemical determination of tocopherol and tocopherylquinone in the feces and are somewhat open to question in view of the possibility of excretion of absorbed tocopherol into the intestinal tract. The use of isotopic tocopherol permits the estimation of absorption by comparison of the rate of fecal excretion, the level of urinary radioactivity, and the nature of the fecal excretion products with the corresponding results obtained on intravenous administration of the material. Such a comparison leads to the conclusion that most of the labeled material recovered in the feces after oral administration, particularly during the first 3 days, must have been unabsorbed vitamin E, since after intravenous injection only 12 per cent of the dose had appeared in the stool during the same period, and since only 40 to 50 per cent of this material was α-tocopherol. A consideration of these results and of the relative levels of urinary radioactivity led us to estimate that the amount of α-tocopheryl succinate absorbed from the gastrointestinal tract in this experiment did not exceed 10 per cent of the oral dose.

It is of interest that following subcutaneous administration the excretion patterns, including the relative amounts of radioactivity in urine and feces, resemble closely those obtained on intravenous injection, except for smaller absolute amounts of daily excretion and a delay of both maxima by 6 days, presumably due to the very slow rate of absorption from the subcutaneous site. Since the amount of tocopherol absorbed averaged approximately 1 mg. per day, or about the quantity an animal may be expected to absorb from its food, this may be taken as evidence that these excretion patterns would hold under physiological conditions.

A direct investigation of radioactivity present in the respiratory carbon dioxide was not feasible because of the low level of radioactivity of the administered material. It seems justified to conclude, in view of the high recovery of radioactivity in urine and feces over such a long period, that oxidation of the carbon atom of the 5-methyl group and its excretion as carbon dioxide can at best be a very minor pathway of elimination.

The data presented indicate that a significant portion of parenterally administered tocopherol is eliminated in some form in the urine and that excretion from the blood stream into the intestinal tract takes place to a large degree. These results bear considerable similarity to the findings of Siperstein and Chaikoff (20) on the elimination by the rat of intravenously administered C^{14}-cholesterol labeled in the steroid nucleus. These authors found that virtually all of the isotopic cholesterol was excreted into the intestinal tract and eliminated in the feces. The period of 15 days required for complete elimination of the administered dose as well as the shape of the fecal excretion curve is strikingly similar to the results reported here for vitamin E. The observation that elimination via the urine is a significant pathway for α-tocopherol, while it is a negligible one for cholesterol, represents the most important difference between the modes of elimination of these two lipide materials.

Nature of Excretion Products Following Parenteral Administration: In Feces—It has already been pointed out that 40 to 50 per cent of the ethanol-extractable fecal excretion products was found to be free α-tocopherol. Preliminary observations on the balance of the fecal radioactivity indicate that it is composed of materials of much more polar character than α-tocopherol, as shown by its distribution between aqueous ethanol and petroleum ether and the behavior of a crude metabolite, isolated from the feces, in reversed phase paper chromatography. This material had an R_F of 0.88 in the system of Eggitt and Ward (14), in which the R_F of α-tocopherol is 0.25. The nature of the fecal excretion products is under investigation.

In Urine—The reason for the negative findings of previous workers looking for urinary α-tocopherol became clear when it was shown by isotope dilution as well as by paper chromatographic procedures that no detectable α-tocopherol or α-tocopheryl succinate was present in urine. On the assumption that small quantities of tocopherol, if they were to appear in the urine, would most likely be present early after administration, when the blood α-tocopheryl succinate level is highest, the urine of one rabbit was collected by catheter during the 6 hours immediately following intravenous injection. The first urine sample obtained 2 hours after injection had the highest specific activity, but, even here, metabolic products of α-tocopherol accounted for 99 per cent of the radioactivity.

Over 90 per cent of the isotopic material in the urine was strongly acidic and could be removed from acidified urine by continuous ether extraction for 48 hours. Continuous extraction with petroleum ether removed less than 10 per cent of the total radioactivity.

Acid hydrolysis of the evaporated ether extracts increased the amount of petroleum ether-extractable isotopic material to 60 to 70 per cent, while incubation with the enzyme β-D-glucuronidase gave a petroleum ether-

soluble radioactive substance in 70 to 90 per cent yield. These results provided evidence that the major excretion product may be a glucuronide.

Since hydrolysis and petroleum ether extraction gave a considerably purified product, as indicated by its spectrum and specific activity, our attention was directed towards a study of this material. Urines of rabbits injected intravenously with massive doses of *d*-α-tocopheryl succinate were used. The petroleum ether extracts of acid or enzyme hydrolysates from the urines of such rabbits showed the characteristic absorption spectrum depicted in Fig. 3. The proportionality of the optical density at the max-

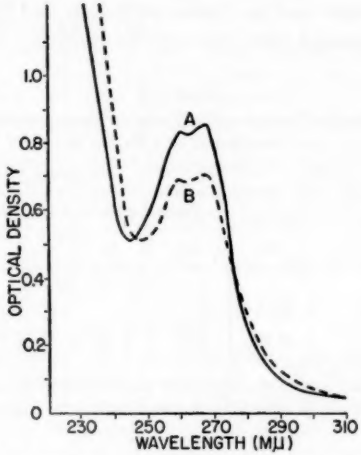

FIG. 3. Ultraviolet absorption spectra of urinary metabolite after hydrolysis and petroleum ether extraction. Curve A, after enzyme hydrolysis; Curve B, after acid hydrolysis.

ima to the specific activity of the sample as well as the similarity of the spectrum to that of α-tocopherylquinone (21), from which it differs only by the presence of end-absorption in the short ultraviolet region, was taken as evidence that the spectrum is really due to the metabolite rather than to impurities. The substance was shown not to be α-tocopherylquinone by its considerably more polar character. While the latter is quantitatively extracted from 50 per cent ethanol by one extraction with an equal volume of petroleum ether, the metabolite distributes itself between these solvents at a ratio of 3:1 in favor of the aqueous ethanol. In the chromatographic system of Eggitt and Ward (14) the metabolite had an R_F of 0.88 compared to 0.50 for α-tocopherylquinone and 0.25 for α-tocopherol. An even more striking demonstration of the vast difference in polarity is shown in Table I,

which gives the R_F values for these compounds in the system of J. A. Brown (15).

Whereas tocopherol and its quinone separated only slightly in this system, the metabolite traveled with the solvent front in concentrations of aqueous acetonitrile in which tocopherol and its quinone did not leave the origin.

Paper chromatography of the hydrolyzed material also indicated the complete absence of a conjugated form of α-tocopherol.

The increase in polarity and the characteristic spectrum of the urinary metabolite obtained on hydrolysis point to oxidation of the chroman ring to the *p*-quinone state and to drastic reduction and possible alteration of the 16-carbon isoprenoid side chain of α-tocopherol. Further purification

TABLE I

Paper Chromatography of α-Tocopherol, α-Tocopherylquinone, and Hydrolyzed Urinary Metabolite of Vitamin E

Compound	R_F value					
	Per cent acetonitrile in developing solvent					
	20	30	40	50	70	90
α-Tocopherol	0	0	0	0	0.29	0.75
α-Tocopherylquinone	0	0	0	0	0.35	0.82
Metabolite	0.60	0.74	0.83	S. F.*	S. F.	S. F.

Whatman No. 1 filter paper impregnated with silicone grease (14) was developed by the ascending technique with the concentrations of aqueous acetonitrile indicated.

* Solvent front.

and characterization of this compound had to await the discovery of a more lucrative source which permitted the accumulation of mg. quantities of this material. This work will be discussed in Paper II of this series (22).

SUMMARY

1. *d*-α-Tocopheryl-5-methyl-C^{14}-succinate was administered to young rabbits by various routes, and its absorption and excretion were studied.

2. Absorption from the gastrointestinal tract and from the subcutaneous site was low when an oily vehicle was used.

3. Complete excretion of radioactivity after intravenous administration required 15 to 20 days. Urine contained 20 to 30 per cent and feces 70 to 80 per cent of the radioactivity recovered from the excreta.

4. The excretion pattern after subcutaneous injection resembled that obtained after intravenous administration, except for a delay of the maxima by 6 days.

5. Free α-tocopherol was found to comprise 40 to 50 per cent of the ethanol-extractable fecal radioactivity after parenteral administration of the tracer dose.

6. The bulk of the urinary radioactivity was shown to be a metabolic product of α-tocopherol. Evidence is presented that it may be conjugated with glucuronic acid. Some of its properties are discussed.

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THE METABOLISM OF VITAMIN E

II. PURIFICATION AND CHARACTERIZATION OF URINARY METABOLITES OF α-TOCOPHEROL*

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Studies with *d*-α-tocopheryl-5-methyl-C^{14}-succinate in rabbits demonstrated the presence of a conjugated metabolite of vitamin E in the urine (2). The product obtained on enzyme or acid hydrolysis, as well as the small amount of unconjugated metabolite excreted, had an ultraviolet absorption spectrum similar to that of α-tocopherylquinone. It was shown, however, to be considerably more polar in nature than the latter, suggesting that rather profound alterations in the structure of α-tocopherol may have taken place.

Urines of humans ingesting large doses of *dl*-α-tocopherol were found to be a good source of a conjugated substance, which on hydrolysis showed spectroscopic, chromatographic, extraction and chemical properties identical with those of the material obtained from rabbit urine. More careful study revealed that two metabolites, one neutral and the other acidic, but both with the same characteristic ultraviolet absorption spectrum (Fig. 1), were obtained on hydrolysis of human urine.[1] The purification and chemical characterization of these metabolites as 2-(3-hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethylbenzoquinone and its γ-lactone will be described. Neither of the metabolites nor the crystalline derivative of the lactone, the preparation and properties of which will be described, has previously been reported in the literature. These structures should, therefore, be considered tentative until definitive proof has been obtained by synthesis.

* This work was supported by Muscular Dystrophy Associations of America, Inc., and by assistance from the Tall Cedars of Lebanon. A preliminary report of this work was presented at the 46th meeting of the American Society of Biological Chemists at San Francisco, April, 1955 (1).

[1] The amount of acidic metabolite present in rabbit urine was so small that it originally escaped notice. It has since, however, been found to be present there as well.

XUM

EXPERIMENTAL

Subjects and Administration—The subjects were persons who were either free from any known disease or had evidence of muscular wasting. During the periods of investigation the subjects were given daily oral doses of 3 to 5 gm. of *dl*-α-tocopherol in gelatine capsules. The urine was collected under careful supervision in 24 hour samples.

Ultraviolet Spectra—Ultraviolet absorption spectra were determined in ethanol purified by distillation over solid potassium hydroxide and potassium permanganate, in silica cuvettes with a 1 cm. light path in a Beckman DU spectrophotometer.

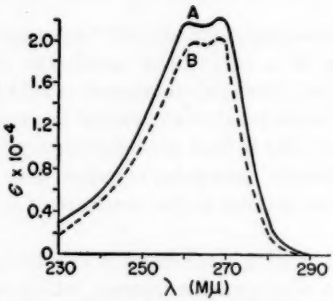


FIG. 1. Ultraviolet absorption spectra of the purified metabolites (Curve A) and of α-tocopherylquinone (Curve B).

Infra-Red Spectra—The infra-red spectra were obtained on a Perkin-Elmer model 21 double beam spectrophotometer. Solvents and prisms used are indicated in Fig. 2.[2]

Isolation and Purification—Urines (24 hour collections) were acidified to pH 1 and extracted continuously with ether for 48 hours. By the procedures described previously for rabbit urine (2) residues from pooled ether extracts were hydrolyzed with β-D-glucuronidase (Warner and Chilcott "ketodase"), and the hydrolysis mixtures were extracted with petroleum ether. Considerable hydrolysis could also be accomplished by letting the wet ether extracts stand at room temperature for several days. Approximately 15 to 30 mg. of crude metabolites per 24 hour urine specimen were obtained in the petroleum ether extracts. This mixture was separated into neutral and acidic fractions by extraction of the solution with aqueous so-

[2] The authors are indebted to the Division of Steroid Metabolism at the Sloan-Kettering Institute for Cancer Research for carrying out the infra-red analyses, and to Miss Friederike Herling and Dr. Glyn Roberts for valuable help in the interpretation of the spectra.

dium bicarbonate. The major portion of material remained in the petroleum ether. The acidic material was recovered by ether extraction of the acidified aqueous solution.

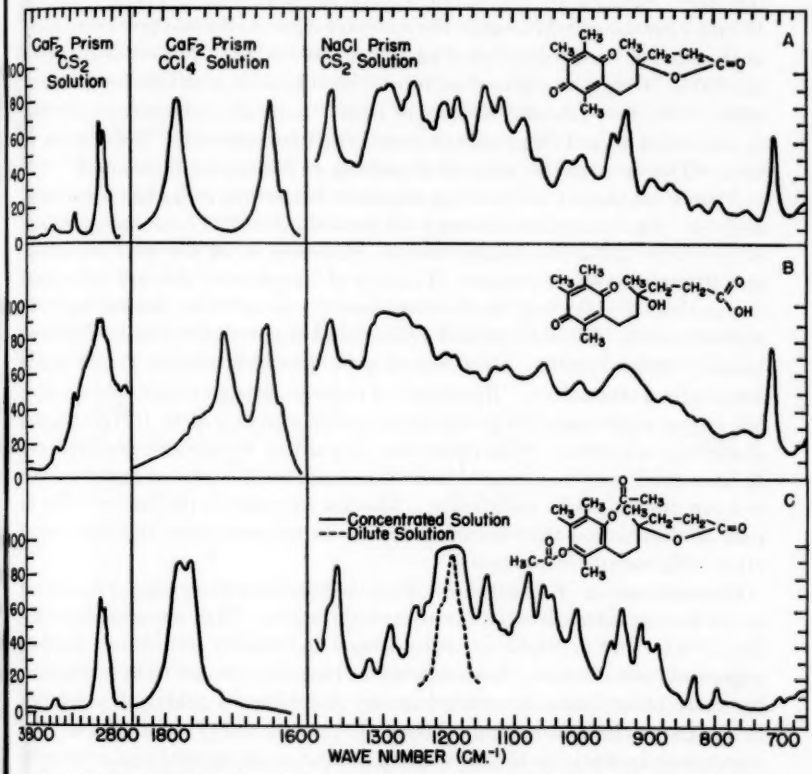


FIG. 2. Infra-red spectra of the metabolites, lactone (A) and hydroxy acid (B), and of the hydroquinone diacetate derivative of the lactone (C) from 4000 to 650 cm.$^{-1}$.

To avoid the acidification and ether extraction steps, enzyme hydrolysis of fresh urine, adjusted to pH 4.5, was tried. It was found unsuitable as a preparative procedure, however, since the products obtained were very crude and were found to be difficult to purify.

Further purification of the metabolites was followed by means of their characteristic ultraviolet absorption (Fig. 1). The optical density at the maxima (260 and 268 mμ) increased with purification, while end-absorption in the far ultraviolet region diminished rapidly. Both materials were puri-

fied by chromatography on a 120 × 20 mm. column of Florex XXS, prepared in ether, by using the gradient elution technique essentially as described by Moore and Stein (3). Tygon tubing is attacked by ether and was, therefore, replaced by polyethylene tubing. The mixing bottle (volume 175 ml.) contained ether, while the reservoir contained a mixture consisting at the start of 7 per cent ethanol and 93 per cent ether. The crude metabolite (50 to 70 mg.) was placed on top of the column in a minimal volume of ether. The flow rate was 30 ml. per hour. 5 ml. fractions were collected in test-tubes in a Technicon automatic fraction collector. Recoveries of 50 to 60 per cent of the material absorbing at 268 mμ were obtained.

A more convenient method that results in better recoveries has since been devised. The procedure utilizes a silicic acid-celite (2:1) column prepared in isooctane and a developing solvent consisting of 90 per cent isooctane and 10 per cent ethyl acetate. The size of the column, the flow rate, and the method of collecting the fractions are the same as in the method described above. The well defined yellow band of metabolite can be followed visually in this system. Recovery of material which absorbs at 268 mμ is essentially quantitative. Repetition of passage through either column does not appear to increase the purity of the product as judged by its ultraviolet absorption spectrum. The quantities of purified metabolites isolated per 24 hour urine specimen ranged from 3 to 6 mg. for the neutral and from 0.5 to 1 mg. for the acidic metabolite. The low recovery of the latter is due in part to destruction that occurred during its removal from the petroleum ether with sodium bicarbonate.

Characterization of Metabolites—Both metabolites are yellow oils which so far have resisted all attempts at crystallization. They are slightly soluble in water and in aliphatic hydrocarbons and readily soluble in all other organic solvents tested. Both substances have been shown to be considerably more polar than α-tocopherol or any of its known oxidation products. In the paper chromatographic system of J. A. Brown (4) with 20 per cent acetonitrile in water as the developing solvent, α-tocopherol and α-tocopherylquinone remain at the origin. The R_F of the neutral metabolite in this system is 0.60 and that of the acidic material is 0.85. These results suggested a reduction in size of the isoprenoid side chain. More conclusive evidence for this is represented by the molecular weight determinations (the Rast method with camphor as the solvent) and by the analyses.[3] For the neutral substance the empirical formula $C_{16}H_{20}O_4$ agrees best with the molecular weight of 267 (256 to 278)[4] and with the analytical results:

$C_{16}H_{20}O_4$. Calculated, C 69.55, H 7.25; found, C 69.34, H 7.29

[3] Analyses and molecular weight determinations were carried out by Dr. Carl Tiedcke, Teaneck, New Jersey.

[4] The molecular weights represent averages of four determinations with a range of values between the limits shown in the parentheses.

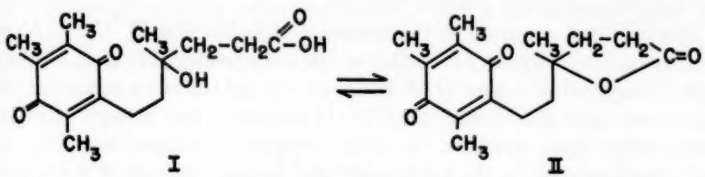
For the acidic material the molecular formula $C_{16}H_{22}O_5$ is most consistent with the molecular weight of 315 (307 to 326) and with the analytical data:

$C_{16}H_{22}O_5$. Calculated, C 65.31, H 7.48; found, C 65.22, H 7.18

These empirical formulae suggest that 13 carbon atoms have been removed from the molecule of α-tocopherol during its metabolism.

Both metabolites, after purification, exhibit the ultraviolet spectrum shown in Fig. 1. This spectrum is qualitatively identical with that of α-tocopherylquinone, and its molar extinction coefficient of 1.9 to 2.2 × 10^4, based on the calculated molecular weights, is in the range of the best values reported for that of α-tocopherylquinone (5). On the assumption that the same chromophoric groups are responsible for these two spectra, the metabolites may be considered to be reasonably pure. This is confirmed by the consistency of the analytical results and infra-red spectra of different samples obtained and purified by the methods described.

The structures postulated for the metabolites are 2-(3-hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethylbenzoquinone (I) and its γ-lactone (II).



I II

Evidence for the various functional groups and the data on the preparation and properties of a crystalline derivative of the lactone will now be presented.

Evidence for Presence of Quinone Grouping—The ultraviolet absorption spectrum (Fig. 1) is characteristic of molecules containing the duroquinone (tetraalkyl *p*-benzoquinone) group, *e.g.* duroquinone (6), dihydrovitamin K (7), and α-tocopherylquinone (5). Further evidence for the presence of a quinone grouping was afforded by the infra-red spectra (Fig. 2, Curve A and Curve B), which for both metabolites showed a C=O stretching band at 1644 cm.$^{-1}$ (CCl_4), characteristic of α,β-unsaturated carbonyl groups (8). This band disappeared on reduction of the quinone.

Evidence for Presence of Carboxyl Group in Acidic Metabolite—The acidic material can be extracted from organic solvents with aqueous sodium bicarbonate which indicates a strongly acid group. Titration with 0.01 N sodium hydroxide in dilute aqueous ethanol with a Beckman pH meter gave a neutralization equivalent of 280 which is in good agreement with the calculated molecular weight and indicates the presence of one acid group per molecule. The pK of 4.9 is in the range of pK values usually observed for carboxyl groups. The carbonyl region of the infra-red spectrum of

this material (Fig. 2, Curve B) shows a band at 1710 cm.$^{-1}$ characteristic of dimeric carboxyl groups and a weaker band at 1755 cm.$^{-1}$ due to the C=O stretching of monomeric carboxyl groups. The hydroxy region shows the broad band from 3500 to below 3000 cm.$^{-1}$ (CS_2) typical of associated O—H stretching found in carboxylic acids (8, 9).

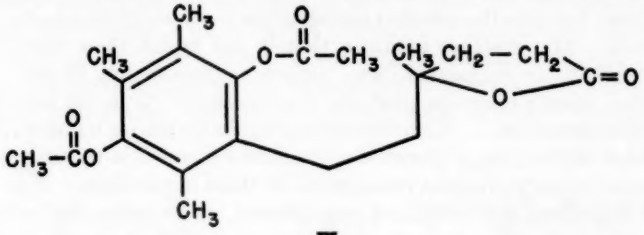
Evidence for Presence of Saturated γ-Lactone Ring in Neutral Metabolite—The neutral metabolite is converted readily into the acidic material by warming with dilute aqueous sodium hydroxide. This conversion is accompanied by considerable loss because of the instability of the metabolites in alkali. The acidic metabolite can be changed into the neutral one by standing for 24 hours with acetic anhydride. This ready interconversion suggested the presence of a lactone, anhydride, or ester grouping. This was confirmed by a positive hydroxamic acid test characteristic of compounds containing such "active acyl groups" (10). The test was quantitative in the best preparations, on the assumption that the color intensity of the hydroxamic acid-ferric ion complex is essentially independent of the types of groups attached to the hydroxamic acid group. The acidic metabolite did not give a positive test.

The infra-red spectrum of the neutral metabolite (Fig. 2, Curve A) was found to differ profoundly from that of the acidic material (Fig. 2, Curve B) in the "finger print" region (1400 to 650 cm.$^{-1}$) and the other regions as well. The broad band due to associated O—H stretching was absent. The carbonyl region again provided the most conclusive information. The 1644 cm.$^{-1}$ band ascribed to the α,β-unsaturated carbonyl groups of the quinone was still present, but the 1710 cm.$^{-1}$ and 1755 cm.$^{-1}$ bands due to the carboxyl group had been replaced by a band at 1780 cm.$^{-1}$. Such a band has been found to be typical of saturated γ-lactones (8, 9) and this band appears to rule out the presence of δ-lactones and esters, which generally absorb at 1750 to 1735 cm.$^{-1}$ (8, 9), anhydrides which have two bands at 1825 to 1800 cm.$^{-1}$ and at 1760 to 1748 cm.$^{-1}$ (9), α,β-unsaturated γ-lactones, which show two bands at 1786 to 1780 cm.$^{-1}$ and 1757 to 1755 cm.$^{-1}$ (8), and β,γ-unsaturated γ-lactones which absorb near 1800 cm.$^{-1}$ (9).

The absence of absorption at 220 mμ in the ultraviolet (Fig. 1) also makes unlikely the presence of an α,β-unsaturated lactone. Finally, unsaturation in the side chain is improbable because of the results of the analysis for hydrogen which would be decreased from 7.25 to 6.57 per cent by the removal of 2 hydrogen atoms. This difference is well outside of the limits of error for the analysis.

Crystalline Derivative of Neutral Metabolite—Reductive acetylation of the lactone with zinc, sodium acetate, and acetic anhydride by the procedure of John and coworkers (5) and recrystallization from iso-

octane yielded a white crystalline compound, m.p. 95–98°. Its proposed structure is that of the lactone of 2-(3-hydroxy-3-methyl-5-carboxypentyl)



III

3,5,6-trimethylhydroquinone diacetate (III). Its molecular weight by the Rast method was 332 (319 to 343).

$C_{20}H_{26}O_6$.	Calculated.	C 66.29, H 7.18
	Found.	" 65.90, " 7.05

The ultraviolet absorption spectrum of this compound is identical with that of α-tocopherylhydroquinone diacetate (Fig. 3), as expected, since the

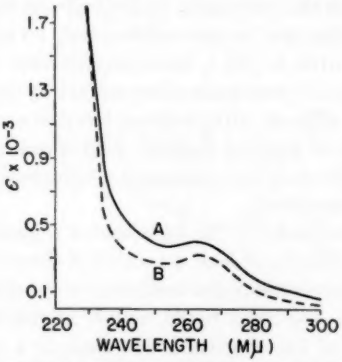


FIG. 3. Ultraviolet absorption spectra of the hydroquinone diacetate derivative of the lactone (Curve A) and of α-tocopherylhydroquinone diacetate (Curve B).

proposed structure would have the same chromophoric groups as the latter. The carbonyl region of the infra-red spectrum (Fig. 2, Curve C) shows that the α,β-unsaturated C=O band at 1644 cm.$^{-1}$ (due to the quinone group) has disappeared. The 1780 cm.$^{-1}$ band ascribed to the γ-lactone C=O is still present and a new band at 1764 cm.$^{-1}$ characteristic of enol acetates (8) has been introduced in agreement with the structure postulated for the derivative.

DISCUSSION

The two metabolites isolated from human urine appear to be identical with those found in the urines of rabbits given *d*-α-tocopheryl-5-methyl-C^{14}-succinate. The evidence indicates that in the rabbit these metabolites, predominantly in conjugated form, comprise approximately 90 per cent of the total urinary excretion products of α-tocopherol (20 to 30 per cent of the intravenous dose). No information is available yet for humans regarding either the fraction of absorbed α-tocopherol excreted in the urine or the portion of urinary products represented by these metabolites. If, in view of the large doses of α-tocopherol administered, we may assume a relatively low level of absorption from the gastrointestinal tract, the amounts of metabolites recovered from the urine represent a fraction of the absorbed vitamin E roughly comparable to the fraction of α-tocopheryl-5-methyl-C^{14}-succinate excreted in the urines of rabbits (2).

The relative amounts of lactone and hydroxy acid recovered may not reflect accurately the actual ratio of these metabolites excreted in view of the destruction of some of the hydroxy acid during sodium bicarbonate extraction[5] and the possibility of interconversion during the isolation procedure. Experiments in which the enzymatic hydrolysis was carried out directly on fresh urine indicate that the lactone is not merely an artifact of storage and ether extraction of urine at pH 1, since considerable quantities of lactone were demonstrated in the petroleum ether extract by paper chromatography (4) and by spectral analysis after sodium bicarbonate extraction. Moreover, when a sample of purified hydroxy acid was stored in aqueous solution at pH 1 overnight and then extracted continuously with ether, no lactone formation was observed.

The structures proposed for the metabolites suggest that the following changes have taken place in at least a portion of the absorbed α-tocopherol: opening of the chroman nucleus and oxidation to the quinone state, shortening of the isoprenoid side chain by 13 carbon atoms, oxidation of the terminal methyl group of the shortened side chain to a carboxyl group, and, in the case of the neutral metabolite, lactonization of the carboxyl group with the tertiary hydroxy group formed as a result of the hydrolytic opening of the chroman nucleus. Another change, not evident in Structures I and II, is conjugation with glucuronic acid and perhaps to a lesser degree with other conjugating groups. The major portion of the urinary metabolites was excreted in conjugated form. The significance of these changes and the possible relationship they may bear to the alterations undergone by

[5] Both metabolites are unstable in alkali as a result of the presence of the quinone group. However, the lactone is not exposed to the destructive action of the alkali since it remains in the petroleum ether.

tocopherol in the formation of its metabolically active form are being investigated.

Work is in progress to determine the structures of the conjugated forms of these metabolites. On the basis of the results of Hoskin *et al.* concerning the nature of the urinary excretion products of menadione (11), it is suggested that the metabolites may be excreted as the corresponding hydroquinones with conjugating groups attached to one or both of the phenolic

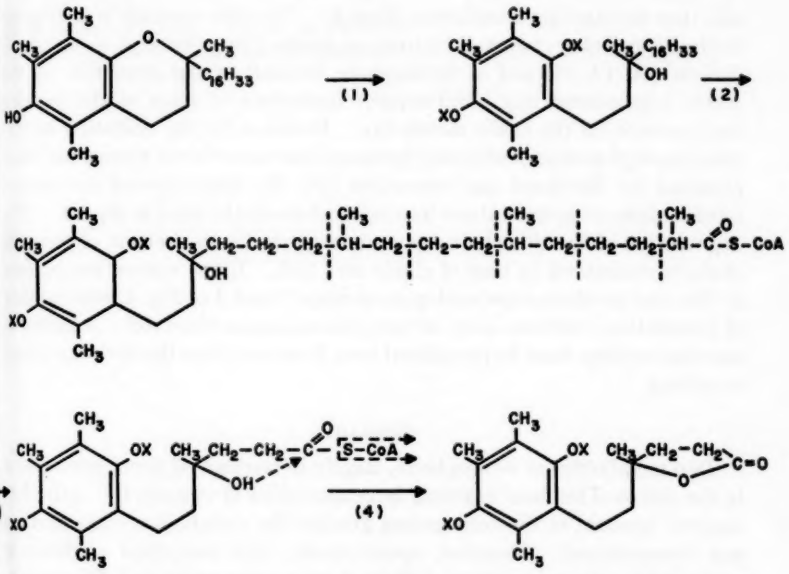


FIG. 4. Hypothetical scheme for the biological formation of the metabolites. X may be a hydrogen atom or a glucuronic acid residue.

hydroxy groups. The hydroquinones formed on hydrolysis would be very susceptible to oxidation by air, forming the observed quinones rapidly.

The same metabolites were obtained from urines of normal humans as from those of patients afflicted with muscular disorders, such as progressive muscular dystrophy, myotonia dystrophica, and amyotrophic lateral sclerosis. No conclusions regarding the relationship, or lack thereof, of vitamin E to muscular disorders can be drawn until quantitative data regarding the excretion of these metabolites become available and until more is known about the relation of these excretion products to the biologically active form of α-tocopherol.

A hypothetical scheme for the biological formation of the metabolites is shown in Fig. 4. In the scheme the chroman ring is opened hydrolytically, and the hydroquinone is conjugated (Step 1). The terminal methyl group of the side chain is oxidized to a carboxyl group, and the resulting fatty acid is conjugated with coenzyme A (Step 2) and degraded by β-oxidation (Step 3). The termination of β-oxidation 2 carbons short of completion may be explained by a nucleophilic attack by the hydroxy group on the acyl coenzyme A to form the stable γ-lactone, pushing out the coenzyme A molecule and thus terminating β-oxidation (Step 4). This last reaction is analogous to the biological acylation of hydroxy or amino groups by acyl coenzyme A derivatives (12, 13) and is, furthermore, favored by the formation of the stable 5-membered ring. Subsequent hydrolysis of some of the lactone may account for the acidic metabolite. Evidence for the oxidation of terminal methyl groups of aliphatic hydrocarbons to carboxyl groups has been obtained by Bernhard and coworkers (14, 15), who injected deuterium-labeled aliphatic hydrocarbons into rats and found the label in the fat. The degradation of the side chain has an analogy in the conversion of the side chain of cholesterol to that of cholic acid (16). If the correct mechanism in this case involves steps analogous to Steps 2 and 3 of Fig. 4, termination of β-oxidation 2 carbons short of completion is again observed. A different termination step must be postulated here, however, since the hydroxy group is missing.

SUMMARY

Two metabolites of α-tocopherol, largely in conjugated form, were found in the urines of humans ingesting large quantities of vitamin E. After hydrolytic removal of the conjugating groups, the metabolites were purified and characterized. Chemical, spectroscopic, and analytical evidence is presented for the formulation of their chemical structures as 2-(3-hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethylbenzoquinone and its γ-lactone. A crystalline derivative of the lactone was prepared. A hypothetical pathway for the biological formation of the metabolites is discussed.

Addendum—Since the proofs were received by us, we have obtained lactone in crystalline form. It crystallizes from 40 per cent ethanol as yellow glistening platelets.

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A CARBON BALANCE ON GLYCINE AND GLUCOSE IN CAROTENE SYNTHESIS

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In a previous paper (1), we reported on the incorporation of the methylene carbon of glycine in carotene in *Phycomyces blakesleeanus* and presented data suggesting that δ-aminolevulinic acid (ALA) had an effect similar to leucine in stimulating carotene production. We now present supplementary evidence on the correctness of the data dealing with labeled glycine by completing a carbon balance with inactive glycine and labeled sugar, together with further experiments on ALA.

We had shown earlier (2) that, in a medium composed of glucose and yeast autolysate, the glucose carbon, comprising 90 per cent of the available carbon in the medium, accounted for less than 40 per cent of the carotene carbon. Subsequently, we reported (3) that incorporation of the α-carbon of leucine was below the 5 per cent level from a medium where the source of nitrogen was a mixture of leucine and asparagine.

EXPERIMENTAL

Inactive Glycine and Randomly Labeled Sugar—A medium was prepared as before (1), consisting of glucose 8 per cent weight per volume, glycine 0.25 per cent weight per volume, inorganic salts, and thiamine (6 mg. per liter). Prior to sterile filtration, about 200 μc. of randomly labeled glucose were added. Twenty-four plates, each containing 20 ml. of medium,[1] were then inoculated with 1 ml. of spore suspension and incubated for 10 days in an air-tight box through which CO_2-free air was drawn. The effluent was passed through 0.1 N NaOH and the CO_2 precipitated as $BaCO_3$ and assayed for activity.

After 10 days, the cultures were harvested, extracted, and saponified. Then the carotenoid extract was chromatographed and the β-carotene separated and crystallized (3) without carrier.

The medium and carotene were assayed for activity as $BaCO_3$ after dry combustion. The specific activities are reported after appropriate corrections have been applied for self-absorption.

[1] We use here a liquid medium without filter paper or supporting Raschig rings. The earlier technique had enabled us to apply ionone or heptenone without spreading of the drop. It is, however, preferable to add ionone, when required, to a small piece of filter paper adhering to the under side of the Petri dish lid.

XUM

Results

The specific activities of medium containing labeled sugar and of the carotene were determined in duplicate. The results for the medium were 752 and 744, and for the carotene 725 and 720 c.p.m. per mg. of $BaCO_3$. The activity of labeled CO_2 was as follows for the 0 to 4 day period and for each successive 24 hour period up to the 10th day: 680, 660, 760, 810, 840, 875, 850 c.p.m. per mg. Unlike results with labeled glycine (1), the activity remained relatively constant throughout the experiment. In conjunction with previous data, a balance sheet may be computed (Table I), indicating the percentage of carbon drawn from the glycine and from the sugar. It is assumed that the thiamine carbon may be ignored, since its contribution cannot exceed 0.01 per cent.

TABLE I

Derivation of Carbon Incorporated into Carotene

Label	Specific activities		Per cent contributed
	Medium	Carotene	
2-C^{14}-Glycine	347*	840*	2.92
1-C^{14}-Glycine	440*	23*	0.06
C^{14}-Glucose	748	722	94.37
Total			97.35

* Data recorded earlier (see (1)).

TABLE II

Effect of Streptomycin on ALA

Nitrogen source	Carotene content		Carotene concentration	
	Streptomycin absent	Streptomycin present	Streptomycin absent	Streptomycin present
	γ	γ	γ per gm.	γ per gm.
Asparagine, 0.25%	455	124	621	212
" 0.25% + leucine, 0.05%	1120	319	1605	511
" 0.25% + ALA, 0.05%	873	295	1295	596

Experiments with ALA—The effect of ALA as a stimulant of carotene synthesis was compared with that of leucine, in the presence and absence of Streptomycin, and its effect was evaluated under limiting conditions of nitrogen supply relative to the available sugar.

Cultures were grown as before, in liquid media, the details of the composition being given in Tables II and III. The Streptomycin was added when indicated, to make a final concentration of 0.05 per cent, after the medium had been autoclaved. Cultures were harvested after 128 hours, and their carotene contents determined spectrophotometrically.

In Table II are presented the amounts of carotene synthesized, and concentrations per gm. of dry mycelium as affected by Streptomycin when grown in a medium containing 2.5 per cent glucose and nitrogen as indicated.

In Table III, the results are given for asparagine-ALA and glycine-ALA mixtures in a 10 per cent glucose solution which shows the yields of dry matter and carotene.

TABLE III

Effect of ALA on Carotene Production

		ALA	Dry matter	Carotene	
	per cent	*per cent*	*gm.*	*γ*	*γ per gm.*
Asparagine	0.20	0.1	0.270	470	1742
	0.20	0.05	0.295	455	1545
	0.20	0	0.317	289	915
	0.10	0.1	0.147	209	1410
	0.10	0.05	0.166	207	1248
	0.10	0	0.192	130	676
Glycine	0.25	0	0.754	1070	1415
	0.25	0.05	0.648	1290	1995

DISCUSSION

From the data in Table I, we have accounted for 97.35 per cent of the carbon in a medium of known composition. The remainder, 2.65 per cent, represents either experimental error or the maximal possible CO_2 fixation. Whichever this may be, 39 of the 40 carbons are accounted for, 38 of which are derived from the glucose, and 1.2 from the methylene carbon of glycine. While the mold preferentially selects the methylene carbon, this cannot represent more than a maximal contribution of 2 carbon atoms to the carotene molecule, even with allowance for a possible contribution from endogenous glycine.

Glycine, therefore, probably does not provide a direct precursor, and the stimulation observed with ALA may also be indirect. If porphyrin and carotenoid syntheses are related, stimulation of the synthesis of one would necessitate a parallel effect on the synthesis of the other.

SUMMARY

When *Phycomyces blakesleeanus* is grown in a medium containing glucose and glycine as the major sources of carbon, the glucose contributes a total of 38 of the carbons of the carotene molecule, and not more than 2 can be derived from the methylene carbon of glycine.

It is therefore deduced that neither glycine nor ALA is likely to furnish direct precursors for carotenoid synthesis, even though the mold preferentially selects the methylene carbon to the extent of a 2-fold enrichment over the medium.

A grant received from the National Science Foundation is gratefully acknowledged.

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CHEMISTRY AND PROPERTIES OF THE 3-ACETYLPYRIDINE ANALOGUE OF DIPHOSPHOPYRIDINE NUCLEOTIDE*

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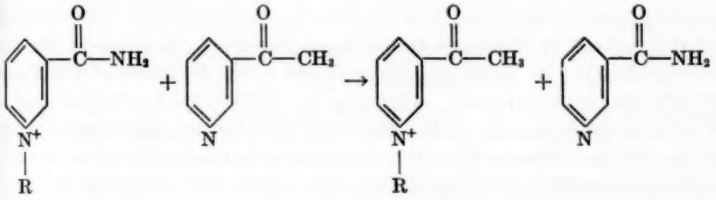
(Received for publication, December 5, 1955)

The DPNase from pig brain has been found to catalyze an exchange reaction between isonicotinic acid hydrazide and DPN to form the isonicotinic acid hydrazide analogue of DPN (1).[1] In a preliminary paper (2) we have reported that the pig brain DPNase catalyzed the formation of the 3-acetylpyridine analogue of DPN (APDPN) according to the following equation, in which R represents the adenosine diphosphate ribose part of the DPN molecule:



DPN + acetylpyridine → APDPN + nicotinamide

The 3-acetylpyridine analogue of DPN is of interest, since it has been shown that 3-acetylpyridine can act as an antagonist of nicotinamide in animals (3–5). It has also been recently demonstrated that the 3-acetylpyridine analogue of DPN is formed in the intact mouse after administration of 3-acetylpyridine to leucemic mice (5). This analogue is the first pyridine-substituted analogue of DPN which has been found to be active in a number of dehydrogenase reactions (6) and also has been proved to be of value in studying enzymatic mechanisms.

This paper compares the chemistry and properties of the 3-acetylpyridine analogue with that of DPN.

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[1] The following abbreviations are used: DPN, diphosphopyridine nucleotide; APDPN, 3-acetylpyridine analogue of DPN; DPNH, reduced DPN; APDPNH, reduced APDPN; Tris, tris(hydroxymethyl)aminomethane.

Materials and Methods

Pig brain, beef spleen, and *Neurospora* DPNases were prepared as described previously (1, 7, 8). Yeast alcohol dehydrogenase was obtained from the Worthington Biochemical Corporation, the 3-acetylpyridine was purchased from the Nutritional Biochemicals Corporation and purified by fractional distillation (9), and the DPN used in these studies was obtained from the Pabst Laboratories, Inc.

Results

Preparation of APDPN—The analogue was prepared by using the pig brain DPNase. The reaction mixture contained 3200 units of the pig brain preparation (a unit is defined as the amount which will cause the splitting of 1 μmole of DPN in 1 hour), 1.25 ml. of 10 M 3-acetylpyridine, 2 gm. of DPN, 6.25 ml. of 1.0 M potassium phosphate (pH 7.5) with water to 150 ml.

To follow the rate of reaction, aliquots were assayed with yeast alcohol dehydrogenase in alcoholic Tris as described previously.[2] The reduced APDPN, as will be discussed below, has a maximal extinction at 365 mμ (see Fig. 3, *A*). By measuring the 365:340 ratio, the levels of DPN and APDPN can be determined. The ratio for DPN is 0.71, whereas that of APDPN is approximately 1.40. Reduced DPN has no significant absorption at 400 mμ, whereas the reduced analogue has a significant extinction at this wave-length (see Fig. 3, *A*). In Fig. 1, the course of APDPN formation from DPN is summarized, and both the change in the 365:340 ratio and the increase at 400 are plotted. The usual conversion of DPN to the analogue is approximately 90 per cent. Maximal APDPN synthesis occurs within 2 to 3 hours. Prolonged incubation leads to a destruction of the APDPN, since acetylpyridine does not inhibit the cleavage of the analogue by the pig brain DPNase.[3]

When maximal synthesis of APDPN is reached, trichloroacetic acid is added to make a concentration of 5 per cent, and the denatured protein is removed by centrifugation. The analogue is then precipitated with 5 volumes of cold acetone. This precipitate usually contains about 5 to 10 per cent unchanged DPN. The DPN was destroyed by the addition of the *Neurospora* DPNase, which does not act on APDPN. This was accomplished by dissolving the precipitate in 50 ml. of 0.1 N NaAc and adding 12,000 units of the *Neurospora* enzyme. The DPN was completely de-

[2] More yeast alcohol dehydrogenase is used in this assay than in the routine assay of DPN, since the rate of reaction of the APDPN is somewhat slower than that of DPN (6). Usually 0.2 ml. of a 1:40 dilution of the enzyme is adequate to determine APDPN.

[3] N. O. Kaplan and M. M. Ciotti, unpublished data.

stroyed in 60 minutes. The reaction mixture was then placed on a Dowex 1 formate column as described by Kornberg and Horecker (10). The APDPN can be eluted from the column by using a mixture of 0.1 M formic acid plus 0.1 M sodium formate in the same manner in which DPN is eluted (10). After precipitating with 5 volumes of acetone, the analogue was washed with acetone and ether and dried. 800 mg. of APDPN were obtained; the purity of the compound based on adenine content was about 90 per cent. No DPN could be detected by assay with the *Neurospora* DPNase, and no free or bound nicotinamide by the Hofmann degradation procedure outlined by Holman (11).

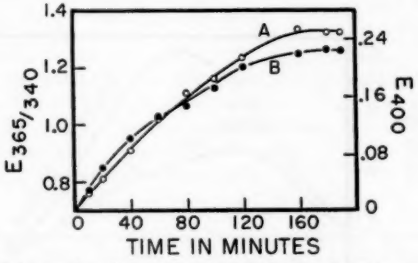


FIG. 1. APDPN formation by pig brain DPNase. In Curve A, E 365/340 refers to the ratio of increase in optical density at these two wave-lengths after addition of yeast alcohol dehydrogenase to aliquots of the reaction mixture described in the text; the time represents the period of incubation with the DPNase. Curve B represents the change at 400 mμ in optical density after addition of yeast alcohol dehydrogenase to aliquots taken at different time intervals. See the text for further details.

Analysis of APDPN—3-Acetylpyridine was identified as a part of the analogue after cleavage with the pig brain DPNase. The 3-acetylpyridine can be separated from the other product of the cleavage (adenosine diphosphate ribose) on a Dowex formate column. Adenosine diphosphate ribose is held tightly on a column, whereas the free acetylpyridine can be washed off the column. The 3-acetylpyridine can be determined by its absorption at 230 mμ (12).

A second procedure to determine 3-acetylpyridine is by its dinitrophenylhydrazone. 3-Acetylpyridine reacts with the reagents of the Friedemann-Haugen method (13) for the determination of keto acids. The rate of reaction of 3-acetylpyridine with dinitrophenylhydrazine is considerably slower than that of pyruvic acid. Hence it was essential to preincubate the dinitrophenylhydrazine with 3-acetylpyridine for a period of 60 minutes at 37° before the addition of the alkali. The dinitrophenylhydrazone of 3-acetylpyridine has a maximum at 450 mμ, and, under the

conditions described above, it is possible to detect from 0.1 to 1.0 μmole of 3-acetylpyridine. APDPN also reacts with dinitrophenylhydrazine, giving a product with a maximal absorption at 500 mμ which is higher than that obtained with the hydrazone of free 3-acetylpyridine (Fig. 2). On treatment of the analogue with the beef spleen DPNase (which liberates free acetylpyridine) a dinitrophenylhydrazone is obtained which is identical to that given by free 3-acetylpyridine.[4] The fact that free and bound acetylpyridines yield different hydrazones suggests that the change to a quaternary ring nitrogen has a pronounced influence on the ketone grouping of 3-acetylpyridine.[5] A summary of the composition of APDPN is given in Table I.

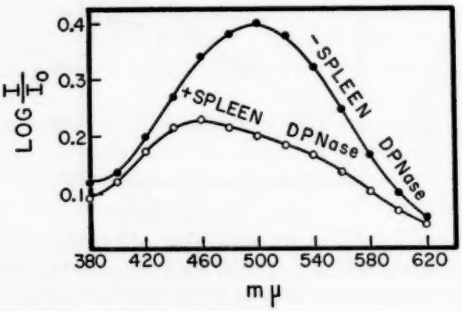


FIG. 2. Spectra of the dinitrophenylhydrazone of APDPN, before and after treatment with the beef spleen DPNase. The curves represent 0.4 μmole of APDPN. 0.2 ml. of purified DPNase was added to liberate the acetylpyridine. The spectrum obtained after treatment with the DPNase is identical to that given by free acetylpyridine.

Spectrum of Reduced APDPN—Fig. 3, *A*, compares the spectra of the reduced analogue with that of DPNH in the range between 290 and 420 mμ. The spectrum was obtained with yeast alcohol dehydrogenase. As can be seen, the maximal absorption of the analogue is at 365 mμ as contrasted to 340 mμ for reduced DPN. The reduced analogue has a millimolar extinction coefficient of 7.8 at 365 mμ as compared to the maximal coefficient of 6.3 for DPNH. An identical spectrum of the reduced analogue was obtained with reduction by hydrosulfite. Yarmolinsky and Colowick (14) have reported that the yellow intermediate formed in the hydrosulfite reduction of DPN is not a half reduced derivative, but probably a

[4] The beef spleen DPNase forms APDPN and can also split APDPN in the same manner as the pig brain DPNase. The beef spleen enzyme was used for liberating acetylpyridine because of its greater potency.

[5] DPN and nicotinamide do not react with dinitrophenylhydrazine.

sulfoxylate addition product of DPN with a maximum between 355 and 360 mμ. APDPN also forms such an intermediate, which is intensely yellow and can be characterized by a distinct peak at 385 mμ.

TABLE I
Analysis of APDPN

Component	Found	Calculated*
	μmoles per mg.	*μmoles per mg.*
3-Acetylpyridine†	1.27	1.37
Total phosphate	2.66	2.74
Adenylic acid‡	1.33	1.37
Ribose§	2.96	2.74

* Calculated on the basis that the compound is 90 per cent pure.

† Determined as the dinitrophenylhydrazone.

‡ Determined with the 5′-adenylic acid deaminase (21), after cleavage of the analogue by the snake venom pyrophosphatase (22).

§ Determined by the orcinol procedure (23).

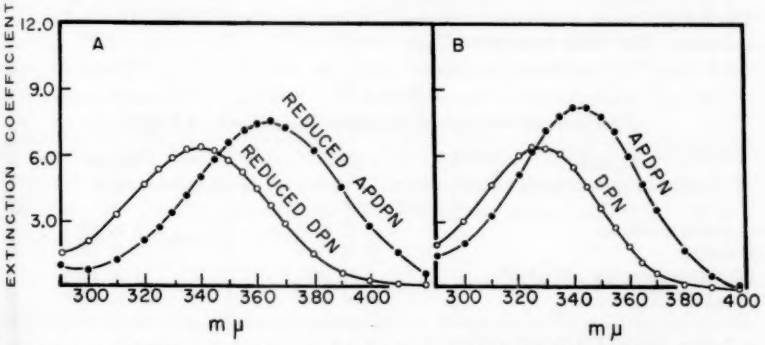


FIG. 3, *A*. Spectra of reduced DPN and reduced APDPN. Both compounds were obtained after reduction with ethanol and yeast alcohol dehydrogenase. *B*, absorption spectra of DPN and APDPN in 1 M KCN.

Reaction of APDPN with Cyanide—DPN reacts with cyanide to give a product with a maximal extinction at 325 mμ (15). APDPN also forms a compound with cyanide, which has a distinct peak at 340 mμ (Fig. 3, *B*). The reaction between APDPN and cyanide is much more favorable than the DPN-cyanide interaction. This is illustrated in Fig. 4; at pH 7.5 with 0.003 M cyanide, there is almost no interaction of DPN with cyanide, whereas there is considerable reaction with APDPN under the same condition. The equilibrium constant of the DPN-cyanide reaction is 1×10^{-7};

the corresponding APDPN reaction is approximately 50 times greater (Table II).

Reaction with Dihydroxyacetone and Bisulfite—APDPN forms addition products with carbonyl compounds such as dihydroxyacetone (16) as well as with bisulfite (15). The maximum of the DPN dihydroxyacetone com-

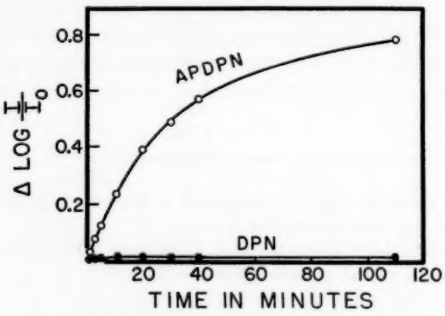


FIG. 4. Reaction of DPN and APDPN in 0.003 M cyanide, 0.1 M Tris (pH 7.5). The APDPN curve represents extinction at 340 mμ, and the DPN curve extinction at 325 mμ. The total volume was 3 ml.

TABLE II

Equilibrium Constants of Reactions of DPN and APDPN

Reaction	DPN	APDPN
	K	*M*
Cyanide addition*	1×10^{-7}	9×10^{-5}
Bisulfite " *	3×10^{-7}	3×10^{-5}
Dihydroxyacetone addition*	3×10^{-7}	1×10^{-5}
Yeast ADH†	1.6×10^{-11}	3.0×10^{-9}

* $K = (\text{DPN}X)(\text{H}^+)/(\text{DPN})(X)$.

† $K = (\text{DPNH})(\text{acetaldehyde})(\text{H}^+)/(\text{DPN})(\text{ethanol})$. See the following paper (6) for the method by which the constant was obtained.

plex is at 340 mμ and that of the corresponding APDPN complex at 350 mμ. The DPN bisulfite product has a maximum of 325 mμ as compared to a 340 mμ peak obtained with APDPN and bisulfite. In Table II the equilibrium constants of the addition reactions of DPN and APDPN are compared. As is the case with the cyanide equilibrium, the APDPN addition reactions with dihydroxyacetone or bisulfite have considerably more favorable equilibria than have the corresponding addition reactions with DPN. Table II also includes data taken from the following paper (6), which shows that the equilibrium with yeast alcohol dehydrogenase is also

more favorable for APDPN. The equilibrium constant for acetaldehyde formation is some 200 times greater with the analogue than with DPN. The data in Table II are strongly suggestive that the equilibria of the addition reactions are related to the oxidation-reduction potentials of the pyridine nucleotides.

Reaction with Alkali—Dilute alkali is known to cleave the nicotinamide ribose link of DPN; APDPN is also split under the same conditions. In 0.1 N NaOH, APDPN gives a reaction characterized by a sharp peak at 330 mμ. After standing in the 0.1 N alkali for 60 minutes, the peak is shifted to a 360 mμ maximum. At the end of this period, there is no detectable intact APDPN, as indicated by reaction with either yeast alcohol dehydrogenase or cyanide.

DPN shows only a slight change in spectrum on the addition of 0.1 N NaOH. However, a definite new peak arises at 290 mμ. The magnitude of the 290 mμ maximum is much lower than the corresponding 330 mμ peak obtained with APDPN. Furthermore, after 60 minutes the 290 mμ peak is absent; no DPN can be detected after this period. The significance of the changes in dilute alkali will be discussed elsewhere.

DPN in the presence of strong alkali forms a product which possesses strong fluorescence (17). This fluorescent compound has been used to determine DPN (17). Under the same condition, however, APDPN does not give a fluorescent product. The difference in fluorescence in 5 N alkali has been used to distinguish DPN from APDPN.

Reaction with Methylethyl Ketone—The reaction of methylethyl ketone with DPN has been used as a basis for the fluorometric determination of DPN (18). In this procedure, APDPN gives about one-third of the fluorescence obtained with DPN.

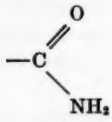
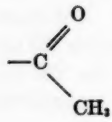
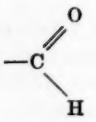
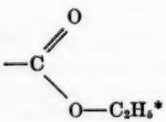
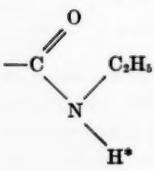
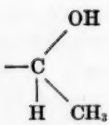
DISCUSSION

APDPN has many properties similar to those of DPN in that it can be reduced, enzymatically or chemically with hydrosulfite, and also forms complexes with reagents such as cyanide, bisulfite, and dihydroxyacetone. The only other analogue of DPN which we have prepared to date that can be reduced enzymatically is the pyridine-3-aldehyde analogue. However, analogues of DPN containing pyridine, β-picoline, or 3-methylpyridyl carbinol cannot be reduced either enzymatically or chemically or react to form addition products. The properties of some analogues of DPN are summarized in Table III. The details concerning these analogues will be published elsewhere.

A comparison of the equilibrium constants of the addition reactions of DPN and APDPN is of interest, since these constants appear to reflect the potential of the pyridine nucleotides. The ratio of the constants APDPN

TABLE III

Comparison of Reactions of DPN with Some 3-Substituted Pyridine Analogues of DPN

	Reaction with cyanide	Reduced by yeast alcohol dehydrogenase	Reduced by hydrosulfite
X			
	+	+	+
	+	+	+
	+	+	+
	+	−	+
	+	−	+
	−	−	−
−CH_3†	−	−	−

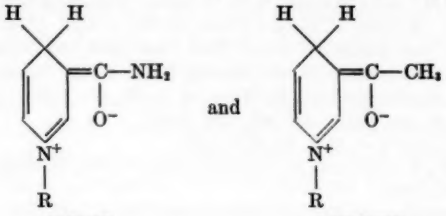
R represents the adenosine diphosphate ribose part of the molecule and *X* the substituent in the 3 position.

* Prepared by Dr. R. M. Burton.

† Prepared by Dr. J. van Eys.

to DPN seems to vary with different reactions (see Table II). Whether this is due to experimental error or an inherent difference in structure of the products is as yet not clear. The addition reactions, nevertheless, appear to be of value in approximating the potential of DPN analogues.

APDPNH has a maximum at 365 mμ as compared to a 340 mμ peak for DPNH; the maximal extinction is also higher for the reduced analogue. These spectral differences may represent a greater tendency for conjugation in APDPNH, since a greater shift to the visible and a higher maximum are usually associated with a conjugated phenomenon (19). It is possible that DPNH and APDPNH can exist in the following conjugated form.



DPNH APDPNH

The fact that a grouping such as $\overset{O}{\overset{\|}{C}}—CH_2$ has a greater capacity to conjugate with rings than does an amide group (20) would be in agreement with the spectral changes which we have observed. It is also possible that the conjugated form is the stable form in which DPNH exists; this stability may be related to the presence of the dipole structure.

SUMMARY

1. The isolation of the 3-acetylpyridine analogue of DPN (APDPN) is described.

2. The reduced analogue (APDPNH) has a peak at 365 mμ with an extinction coefficient of 7.8×10^6 sq. cm. per mole.

3. APDPN reacts with cyanide, bisulfite, and dihydroxyacetone to form complexes which can be characterized by maxima at 340, 340, and 325 mμ, respectively. The association of APDPN with these reagents is much more favorable than with DPN.

4. The properties of APDPN and DPN are discussed with respect to their structural differences.

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REACTION OF PYRIDINE NUCLEOTIDE ANALOGUES WITH DEHYDROGENASES*

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(Received for publication, December 5, 1955)

In the preceding paper (1), the properties of the 3-acetylpyridine analogue of DPN (APDPN)[1] have been described. The fact that the analogue can be reduced made it of value to investigate in some detail the enzymatic activity of this analogue. The present manuscript is the first of a series which deals with the enzymatic activity of pyridine-substituted DPN analogues. Data will be presented which demonstrate that not only does APDPN react with dehydrogenases, but in some cases actually reacts faster than DPN. Some information will be given on the pyridine-3-aldehyde analogue of DPN (Py3A1DPN), which also can be reduced enzymatically.

Materials and Methods

Coenzymes—APDPN was prepared as described in the preceding paper (1), and Py3A1DPN was prepared in the same manner as APDPN.[2] This analogue has a reduced band at 355 mμ, with an extinction coefficient of 7.5×10^{-6} sq. cm. per mole. The ethyl nicotinate[3] analogue was also prepared by the exchange reaction with the pig brain system; the reduced ethyl nicotinate analogue has a maximal absorption at 340 mμ and the same extinction at this wave-length as reduced DPN. APTPN was isolated in the same fashion as was APDPN, with the reaction between TPN and acetylpyridine in the presence of the pig brain enzyme. APTPNH, prepared either by hydrosulfite or enzymatic reduction, has the same maximal

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[1] The following abbreviations are used: ADH, alcohol dehydrogenase; Py3A1DPN, pyridine-3-aldehyde analogue of DPN; DPN, diphosphopyridine nucleotide; APDPN, 3-acetylpyridine analogue of DPN; TPN, triphosphopyridine nucleotide; APTPN, 3-acetylpyridine analogue of TPN; DPNH, reduced DPN; Tris, tris(hydroxymethyl)aminomethane.

[2] Pyridine-3-aldehyde was obtained from the Aldrich Chemical Company, Inc.

[3] Ethyl nicotinate was obtained from the Eastman Organic Chemicals Department, Division of the Eastman Kodak Company.

extinction at 365 mμ as does APDPNH. TPN and DPN were obtained from the Pabst Laboratories, Inc.

Enzymes—Yeast alcohol dehydrogenase was purchased from the Worthington Biochemical Corporation; horse liver alcohol dehydrogenase was crystallized by the procedure of Bonnichsen (2). The liver glutamic acid dehydrogenase was a crystalline protein which was prepared by Strecker's method (3); Straub's procedure (4) was used in obtaining a crystalline lactic dehydrogenase from beef heart, and the rabbit muscle lactic dehydrogenase was prepared by the procedure of Kornberg (5). Muscle triosephosphate

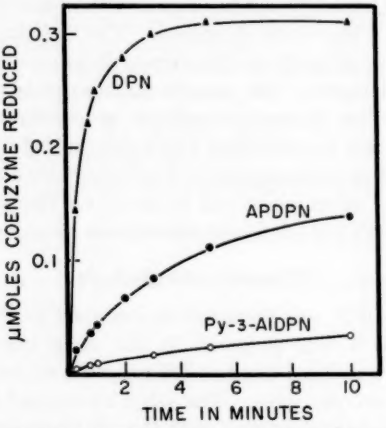


FIG. 1. Rate of reduction of DPN and the analogues by yeast ADH. Reaction mixtures contained 0.3 μmole of nucleotide in each case, 0.1 M Tris, pH 10, and 0.5 M alcohol in a total volume of 3 ml. The reaction was started by the addition of 40 γ of crystalline yeast ADH.

dehydrogenase was obtained by the method of Cori *et al.* (6); crystalline yeast triosephosphate dehydrogenase was a generous gift of Dr. Gale Rafter and was prepared as outlined by Krebs *et al.* (7). The TPN isocitric dehydrogenase was the partially purified preparation described by Grafflin and Ochoa (8).

Results

Reaction of Analogues with Yeast ADH—The comparative rates of reaction of several analogues of DPN with that of DPN are given in Fig. 1. APDPN reacts at approximately one-tenth the rate of DPN. Py3A1DPN reacts considerably more slowly than APDPN, being reduced at about 2 per cent of the DPN rate. The ethyl nicotinate analogue of DPN which can be reduced with hydrosulfite is not reduced in the yeast

ADH system. The K_m for APDPN and for Py3A1DPN was found to be approximately the same as that of DPN.

Although APDPN reacts at a slower rate than does DPN, the equilibrium is much more favorable for the reduction for APDPN than for DPN.

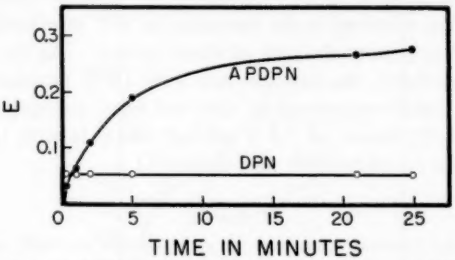


FIG. 2. Reduction of DPN and APDPN at pH 6.6 by yeast ADH. The reaction mixtures contained 0.1 M phosphate (pH 6.6), 0.016 M ethanol, 200 γ of either DPN or APDPN in a total volume of 3 ml. 40 γ of yeast ADH were added to initiate the reaction. *E* represents the optical density change for DPN at 340 mμ and for APDPN at 365 mμ.

TABLE I

Comparison of Equilibrium Constants Obtained with Yeast ADH for DPN and APDPN at Different Hydrogen Ion and Ethanol Concentrations*

Ethanol	H^+	DPN	APDPN
M	*M*	*K*	*K*
1.6×10^{-3}	3.1×10^{-7}	*	3.8×10^{-9}
1.6×10^{-2}	3.1×10^{-7}	1.5×10^{-11}	4.2×10^{-9}
3.3×10^{-4}	4.0×10^{-8}	0.8×10^{-11}	1.7×10^{-9}
1.6×10^{-3}	4.0×10^{-8}	1.3×10^{-11}	1.2×10^{-9}
3.3×10^{-4}	8.5×10^{-9}	3.0×10^{-11}	4.1×10^{-9}
Average		1.6×10^{-11}	3.0×10^{-9}

* The amount of DPN reduced at this hydrogen ion and ethanol concentration is too low to make an accurate calculation.

This is illustrated in Fig. 2, where at a low ethanol level and at pH 6.6 there is practically no formation of DPNH; however, a considerable reduction of APDPN still occurs. Table I summarizes the data obtained for the yeast ADH equilibrium constants with DPN and APDPN. The average *K* with DPN was found to be 1.6×10^{-11}, a value in good agreement with that obtained by Racker (9). The average equilibrium constant found with APDPN was almost 200 times as great (3.0×10^{-9}).[4]

[4] Almost an identical equilibrium constant was obtained when APDPNH and acetaldehyde were the reactants.

This certainly indicates a significant difference in potentials between APDPN and DPN. Preliminary data with Py3A1DPN indicate that the potential of this analogue is close to APDPN.

Reaction with Horse Liver ADH—APDPN reacts much faster with the horse liver ADH than does DPN (Table II). This difference depends on the concentration of ethanol; for example, at 0.01 M ethanol, DPN is reduced at about one-third the rate of the analogue. As the concentration of ethanol is increased, the reaction rate with DPN decreases. However, elevating the alcohol concentration does not have as marked an effect on APDPN reduction; hence, at 2.5 M alcohol, the rate with the analogue is 10 times as great as with DPN (see Table II).

TABLE II

Effect of Alcohol Concentration on Rate of Reduction of DPN, APDPN, and Py3A1DPN by Horse Liver ADH

The reaction mixtures contained 0.1 M Tris, 200 γ of nucleotide in each case, and the alcohol concentrations indicated below. The reaction was started with 60 γ of the crystalline liver enzyme. The values represent the rate expressed as micromoles of nucleotide reduced × 1000 for the first 15 seconds of the reaction.

Ethanol concentration	DPN	APDPN	Py3A1DPN
M			
0.01	35	103	12
0.1	22	129	21
0.5	17	92	25
2.5	9	85	22

Py3A1DPN reacts at a slower rate with the liver dehydrogenase than does DPN at low alcohol levels. Table II indicates that the concentration of ethanol does not influence the rate of formation of Py3A1DPNH and actually at the higher levels of substrate the Py3A1DPN reacts more rapidly than DPN. It appears that the effect of a high level ethanol on the horse liver ADH is a complex phenomenon, and that factors other than protein denaturation are involved. It is of interest that, at low concentrations of ethanol (0.01 M), the rate with APDPN and Py3A1DPN is slower than at higher levels of substrate (0.1 M). The ethyl nicotinate analogue of DPN is also inactive in the liver alcohol dehydrogenase system.

In Fig. 3 is compared the affinity of the analogues with that of DPN for the horse liver ADH. The K_m for DPN was found to be 0.3×10^{-4} and that of APDPN 1.4×10^{-4}. Py3A1DPN had a considerably lower affinity, as indicated by a K_m of 2.5×10^{-4}. Although the analogues have higher K_m values than DPN, the maximal rates are higher with the analogues.

Analogue Activity with Lactic Dehydrogenases—Representative curves are

presented in Fig. 4 on the rate of reaction of APDPN and Py3A1DPN with beef heart and rabbit skeletal muscle lactic dehydrogenases. In the case of the beef heart enzyme, APDPN reacts at about one-tenth of the rate of DPN. Py3A1DPN reacts at a considerably faster rate than

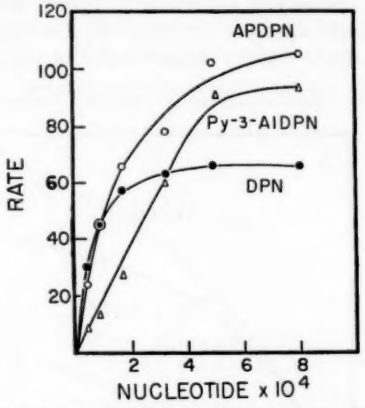


FIG. 3. Effect of pyridine nucleotide concentration on the reaction rate of horse liver alcohol dehydrogenase. Reaction mixtures contained 0.003 M ethanol, 0.1 M Tris, pH 10, and varying levels of DPN or analogues. Reaction started with 60 γ of the horse liver enzyme. "Rate" represents micromoles of nucleotide reduced times 1000 from 15 to 30 seconds after addition of enzyme.

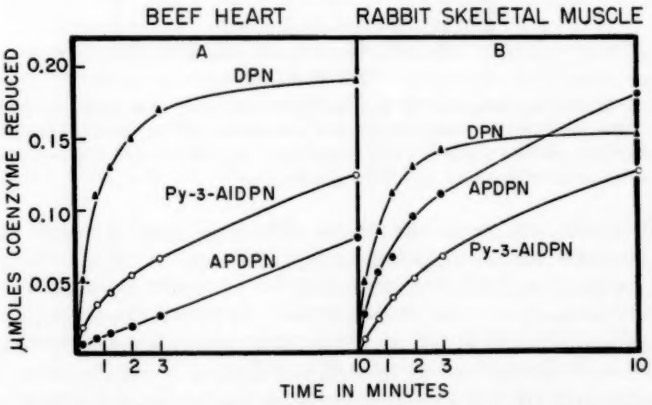


FIG. 4. Rate of reaction of DPN and analogues with lactic dehydrogenases. *A*, reaction mixture contained 0.1 M lithium lactate, 0.1 M phosphate, pH 9, and 200 γ of nucleotide in each case in a total volume of 3 ml. Reaction was started with 11 γ of the beef heart enzyme. *B*, same as *A*, but with 23 γ of rabbit skeletal muscle lactic dehydrogenase.

APDPN with this enzyme and is reduced at one-third the rate of DPN. In contrast, APDPN reacts considerably more rapidly than does Py3A1DPN with the skeletal muscle enzyme, at a rate of about 60 per cent of DPN. The striking difference in the rates of reaction of the two analogues with the two lactic dehydrogenases appears to be of considerable significance, particularly since the two enzymes have been found to possess many similar properties. The K_m values for the two analogues are roughly the same as for DPN for both lactic dehydrogenases.

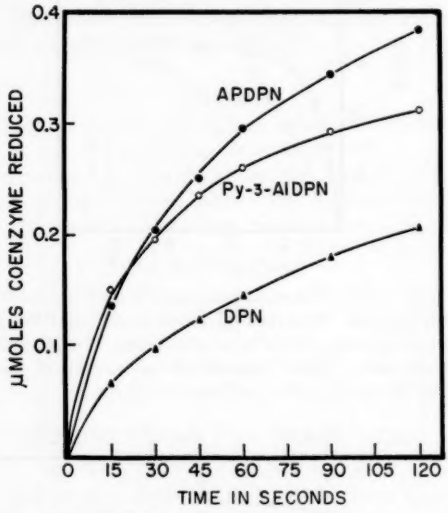


FIG. 5. Rate of reaction of DPN and analogues with beef liver glutamic dehydrogenase. Reaction mixtures contained 0.05 M phosphate, pH 7.6, 200 γ of the glutamic dehydrogenase, and 0.5 μmole of DPN or analogue in a total volume of 3 ml. Reaction was initiated with 0.3 ml. of 0.375 M glutamate.

The equilibrium with the lactic dehydrogenases is again much more favorable for the reduction of APDPN than of DPN. Using the heart enzyme, we have found that K = ((DPNH)(pyruvate)(H^+))/(((DPN^+)(lactate)) = 1.2×10^{-12}, and K = ((APDPNH)(pyruvate)(H^+))/((($APDPN^+$)(lactate)) = 2.7×10^{-10}. The constants are an average of a number of determinations at different hydrogen ion concentrations. Our value with DPN is slightly lower than that obtained by Racker (9). The equilibrium values, again, indicate the more positive potential of APDPN.

Reaction of Analogues with Glutamic Dehydrogenase—Both APDPN and Py3A1DPN react at a faster rate with the liver glutamic dehydrogenase

than does DPN (Fig. 5). The curves in Fig. 5 were obtained with 0.03 M glutamate; when low levels of glutamate (1×10^{-3} M) are employed, the rate is slightly faster with DPN.

Reduction of the two analogues proceeds much further towards completion than DPN does, when low amounts of glutamate are present. Fig. 6 shows that with both APDPN and Py3A1DPN the reduction is almost stoichiometric with the added glutamate. The curves in Fig. 6, again, in-

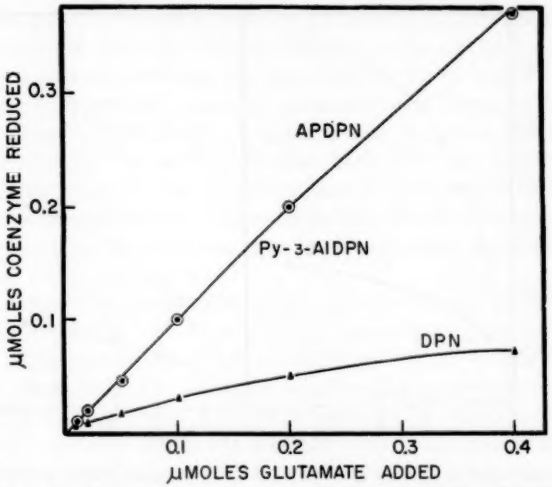


FIG. 6. Relationship of glutamate concentration to DPN and DPN analogue reduction. Reaction mixture contained 0.05 M phosphate, pH 7.6, 0.5 μmole of DPN or analogue, and 400 γ of glutamic dehydrogenase in a total volume of 1 ml. Reaction was started with varying amounts of potassium glutamate as indicated in the graph. The values represent the final extent of the reaction. Solid triangle, DPN; open circle with dot, APDPN or Py3A1DPN.

dicate the more favorable equilibrium for the analogues. For DPN, we have obtained an average K for the reaction[5] of 0.61×10^{-13}, which is close to the value reported by Strecker (3), and the K with APDPN was found to be approximately 1000 times greater ($K = 0.56 \times 10^{-10}$). This more favorable equilibrium is suggested by the data in Fig. 6, in which small levels of glutamate appear to be quantitatively oxidized by APDPN and Py3A1DPN. The ratio of the constant APDPN to DPN seems to be significantly different in the glutamic dehydrogenase reaction from that obtained for the alcohol or lactic dehydrogenase system.

[5] K = ((DPNH) (α-ketoglutarate) (NH_3^+) (H^+))/((DPN) (glutamate) (H_2O)). In this calculation we have used the standard concentration of H_2O as 1 M.

Reaction with Muscle and Yeast Triosephosphate Dehydrogenases—Fig. 7 compares the activity of the analogues with DPN in the two triosephosphate dehydrogenase reactions. It is of interest that APDPN reacts with both enzymes, whereas Py3A1DPN shows no observable activity. Py3A1DPN also markedly inhibits the reduction of DPN by 3-phosphoglyceraldehyde (see Fig. 7). Free pyridine-3-aldehyde has no effect on the formation of DPNH in either triosephosphate dehydrogenase system.

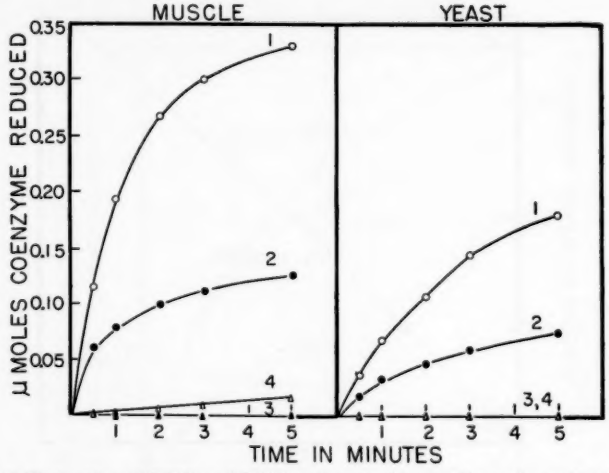
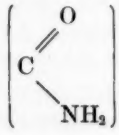


FIG. 7. Reduction of DPN and DPN analogues by muscle and yeast triosephosphate dehydrogenases. Reaction mixtures contained 0.1 M sodium pyrophosphate, pH 8.5, 0.17 per cent sodium arsenate, and 4 γ of muscle triosephosphate dehydrogenase or 16 γ of yeast enzyme in a total volume of 3 ml. The reaction was started by adding 0.54 μmole of phosphoglyceraldehyde. Curve 1, 500 γ of DPN; Curve 2, 500 γ of APDPN; Curve 3, 500 γ of Py3A1DPN; and Curve 4, 500 γ of DPN + 500 γ of Py3A1DPN.

Reaction of APTPN with TPN Isocitrate Dehydrogenase of Pig Heart—The rate of reduction of APTPN in the TPN isocitrate system is about one-fifth that of the natural coenzyme. APDPN is inactive with this TPN-specific enzyme.

DISCUSSION

It is evident from the results of this paper that the nicotinamide



group is not essential for DPN activity. Both APDPN and Py3A1DPN react with dehydrogenases; the rate of reaction of the analogue varies considerably with different enzymes. Although APDPN reacted with all the enzymes reported in this study, the analogue showed no detectable activity in the hexitol phosphate dehydrogenase from *Escherichia coli* (10).

Comparison of the rates of reaction of APDPN and DPN with the horse liver and yeast alcohol dehydrogenases is of interest. APDPN reacts at approximately one-tenth the rate of DPN with the yeast system, whereas the analogue reacts at a faster rate with the liver dehydrogenase. The rate of reduction of DPN by the liver enzyme is diminished by high concentrations of ethanol, whereas the rate with APDPN and Py3A1DPN does not change appreciably when the alcohol level is increased (see Table II). In the case of Py3A1DPN, it appears that higher concentrations of ethanol are required for saturation than with DPN or APDPN. The effect of alcohol concentration on the activity of the horse liver enzyme is not clear at present; however, it is possible that this phenomenon may be related to the substrate-coenzyme complex intermediate previously postulated for the ADH reaction (11).

It is of interest that the affinities of the analogues for yeast ADH are the same as that for DPN. However, the analogues have lower affinities for the liver ADH. Although the K_m values are higher for APDPN and Py3A1DPN than for DPN, the rates at saturation levels are considerably faster for the analogues with the liver system. The reason for the above observations is certainly not clear, although it may be related to the finding that the liver protein binds DPNH and the yeast enzyme does not (12). It is possible that the lower activity of DPN at saturation levels with the liver dehydrogenase is also connected to the binding mechanisms.

Py3A1DPN appears to act in most instances more slowly than either DPN or APDPN. In the case of glutamic dehydrogenase, however, both APDPN and Py3A1DPN react at the same rate and faster than DPN at high concentrations of glutamate. It is of interest that Py3A1DPN is reduced more rapidly than APDPN by the beef heart lactic dehydrogenase, whereas the skeletal muscle enzyme catalyzes a faster reduction of APDPN. The difference in response of the two analogues to the two lactic dehydrogenases suggests the possibility that subtle differences exist in the mechanism of action or in the structure of the two proteins. Further studies are now in progress on the use of coenzyme analogues to determine differences in mechanism and structure of proteins catalyzing the same reaction.

No detectable activity was found with Py3A1DPN with either the muscle or yeast triosephosphate dehydrogenases; APDPN was active with both enzymes. The inactivity of Py3A1DPN with the triosephosphate dehydrogenases may be particularly significant, since in all the other reac-

tions studied both APDPN and Py3A1DPN were active, although the degree of reactivity of the two analogues appears to vary with different dehydrogenases. Py3A1DPN is not only inactive in the triosephosphate dehydrogenase systems, but is also a potent inhibitor of DPN reduction. The inhibition appears to be competitive.

The equilibrium constants obtained for yeast ADH and the beef heart lactic dehydrogenase with APDPN are 200 times as great as that obtained with DPN. However, APDPN in the glutamic dehydrogenase system gave a constant 1000 times as great as that with DPN. The reason for this discrepancy is not clear. We have not ruled out that the more favorable equilibrium with APDPN in the glutamic system is the result of secondary reactions involving ammonia (or the imino acid) with the coenzyme analogues. If we assume that the equilibrium values for ADH and the lactic dehydrogenases are the more accurate indicators of the comparative equilibrium between DPN and APDPN, the oxidation-reduction potential of the analogue can then be calculated as follows:

$$\Delta F^\circ = RT \cdot \log K_1, \qquad K_1 = \frac{K \cdot \text{APDPN}}{K \cdot \text{DPN}} = 200$$

Therefore ΔF° at 30° = 3300 calories. Hence, the potential span between APDPN and DPN would be 3300/46,136 or 0.072 volt. By using an E'_0 value of −0.320 volt for the DPN-DPNH potential (given by Burton and Wilson (13) and Rodkey (14)), the potential of the APDPN-APDPNH system would be −0.248 volt. If the glutamic dehydrogenase constants are used, E'_0 for the analogue would be −0.226 volt. Since the values with the glutamic dehydrogenase may be erroneous, we would like to assign a value of −0.248 volt for the average standard potential of APDPN-APDPNH. The potential for the Py3A1DPN system appears to be quite close to the E'_0 value of APDPN. The curves in Fig. 6 suggest that, by using APDPN, glutamate can be determined quantitatively with glutamic dehydrogenase. Preliminary studies in this laboratory indicate that the more favorable equilibria obtained with APDPN make assay methods for lactate and ethanol, as well as glutamate, much more feasible than when DPN is the electron acceptor. The use of analogues for the determination of various compounds will be described in detail elsewhere.

DPN analogues substituted in the pyridine moiety also have been found to be of value in studying mechanisms of binding of coenzymes to dehydrogenases, in studies of electron transport, and in elucidating possible pathways of oxidative phosphorylation. Further reports from this laboratory will deal with these topics.

SUMMARY

1. A comparison of the activities of the 3-acetylpyridine and pyridine-3-aldehyde analogues of DPN (APDPN and Py3A1DPN) with DPN in a number of dehydrogenase systems is presented.

APDPN reacts with yeast alcohol dehydrogenase at approximately 10 per cent the rate of DPN; Py3A1DPN reacts at a considerably slower rate. With horse liver alcohol dehydrogenase, APDPN and Py3A1DPN react more rapidly at saturation levels than does the natural coenzyme. The K_m values for the two analogues are approximately the same as that of DPN with the yeast alcohol dehydrogenase. However, the affinities of the two analogues for the horse liver enzyme are considerably lower than that of DPN.

Py3A1DPN is reduced by beef heart lactic dehydrogenase at a faster rate than APDPN, whereas with the same enzyme from rabbit skeletal muscle the reaction is more favorable with APDPN.

Both APDPN and Py3A1DPN react at faster rates than DPN with beef liver glutamic dehydrogenase. APDPN reacts with both muscle and yeast triosephosphate dehydrogenases; however, Py3A1DPN shows no measurable activity with these two dehydrogenases and is in fact a potent inhibitor of both enzymes.

The acetylpyridine analogue of TPN (APTPN) reacts with the TPN isocitrate dehydrogenase system from pig heart at about 20 per cent the rate of TPN. APDPN is inactive in this reaction.

2. The equilibrium constant for APDPN is some 200-fold greater with yeast alcohol dehydrogenase and beef heart lactic dehydrogenase than with DPN. The equilibrium constant with glutamic dehydrogenase is approximately 1000 times more favorable for the reduction of APDPN over DPN. An average potential of -0.248 volt has been assigned to the APDPN-APDPNH system as compared to an E_0' of -0.320 volt for DPN-DPNH. The oxidation-reduction potential of Py3A1DPN appears to be close to that of APDPN.

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THE FATE OF THE METHYL GROUP IN 2-METHYL-C^{14}-AMINOFLUORENE, INCLUDING STUDIES OF THE CARCINOGENICITY OF 2-METHYL- AND 2-DIMETHYLAMINOFLUORENE*

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4-Dimethylaminoazobenzene and related compounds exert a powerful and specific carcinogenic action on the livers of rats (1). This effect depends to a considerable extent upon the presence of at least one methyl substituent on the amino nitrogen. Metabolic studies (2, 3) have shown, however, that dealkylation is an important step in the biochemical transformations of this class of compounds. On the other hand, 2-dimethylaminofluorene (2-Me_2AF) as well as the dealkylated products 2-methylaminofluorene (2-MeAF) and 2-aminofluorene (2-AF) all exhibit carcinogenic activity (4, 5). It was, therefore, of some interest to investigate the metabolism of these compounds with special reference to the fate of the methyl group and the possible reversible conversion of the tertiary and secondary alkyl amine to the primary amine. The study was carried out in normal rats by using 2-methyl-C^{14}-aminofluorene (2-Me-C^{14}-AF) to trace the methyl carbon atom. Parallel experiments were performed in riboflavin- and pantothenic acid-deficient rats to determine the effect of lower cofactor levels on the metabolic conversion of the methyl group, and thereby gain information on the mechanism of the reaction.

Incidental to these biochemical studies, the carcinogenic effects of 2-MeAF and of 2-Me_2AF were reexamined in Buffalo strain female rats. Contrary to the results reported by previous investigators (4, 5), an appreciable yield of hepatomas was obtained.

Materials and Methods

The metabolic studies were undertaken with 2 to 3 month-old female Buffalo rats. Some animals were placed on a complete diet, and some on vitamin-deficient semisynthetic diets, as follows: vitamin-free casein

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XUM

(Labco) 360 gm., cerelose 390 gm., modified McCollum No. 185 salt mixture 45 gm., lard (fortified with vitamins A and E) 10 gm., lard 190 gm., and choline 5 gm. A vitamin mixture, consisting of folic acid 10 mg., biotin 1 mg., pyridoxine hydrochloride 20 mg., thiamine 40 mg., riboflavin 10 mg., niacin 200 mg., and calcium pantothenate 140 mg., was added to a portion of the cerelose prior to incorporation in the diet mixture. Omission of the riboflavin or calcium pantothenate from the mixture gave the corresponding deficient diets. Signs of severe riboflavin or pantothenic acid deficiencies were present in 7 to 9 and 12 to 15 weeks, respectively.

2-Me-C^{14}-AF was obtained from Dr. F. E. Ray (6). The radioactive compound was fed by stomach tube in a 0.4 N hydrochloric acid solution containing a few drops of ethanol at a level of 10 mg. per 100 gm. of body weight. The animals were kept in glass metabolism cages designed to allow separate collection of respiratory CO_2, urine, and feces. Food and water were available *ad libitum*. At the end of 72 hours, the animals were sacrificed under ether anesthesia by withdrawing blood from the abdominal aorta into a heparinized syringe. The organs were removed and dried *in vacuo* prior to the determination of radioactivity by combustion.

The carcass and an aliquot of the fresh liver were homogenized with ethanol in a Waring blendor. Choline was determined in the ethanol extracts of the minced carcass or liver. By the procedure of du Vigneaud *et al.* (7), choline was isolated as the reineckate. The choline reineckate was decomposed and the choline precipitated as the phosphotungstate, which was recrystallized from 50 per cent ethanol to constant specific activity. The crude protein residue from the ethanol extractions was treated as described by Miller *et al.* (2) for serine determination. Aniline was used to remove excess HCl from the protein hydrolysate (8) and the serine crystallized to constant specific activity from water-ethanol. The purity was checked by chromatographing a sample on paper with the solvent system *tert*-butanol-formic acid-water, 70:15:15 volume per volume. The serine and choline were degraded to formaldehyde or trimethylamine, rspectively, by the standard procedures (7, 9) and the radioactivity of these fragments was determined. Creatinine was isolated as the picrate from the urine and crystallized to constant specific activity as potassium creatinine picrate.

Urines of all the rats were chromatographed on paper in the three different solvent systems described earlier (10), and autoradiographs were made of the developed chromatograms.

The excretion of unchanged 2-Me-C^{14}-AF in the urine was determined by carrier isotope dilution methods. In a typical experiment, 0.5 to 1.0 ml. of urine containing 300,000 to 500,000 c.p.m. was added to 200 to 230 mg. of unlabeled MeAF dissolved in 5 ml. of 0.4 N HCl. After mixing, the

free amine was precipitated with ammonium hydroxide. The amine was chromatographed in benzene solution on an alumina column, then recrystallized five times as the hydrochloride from ethanol-ether. After conversion to the free amine, the compound was allowed to react with 3,5-dinitrobenzoyl chloride in benzene solution to yield the corresponding crude amide, m.p. 163–164°. This derivative was chromatographed in benzene solution on an alumina column and recrystallized from ethanol three times to give yellow needles, m.p. 166–168°.

$C_{21}H_{15}O_6N_3$. Calculated, C 64.78, H 3.88; found, C 64.96, H 3.86

The specific radioactivity per millimole of the material was determined at each step by plating an aliquot on glass disks until constancy was achieved in three successive operations. The amount of labeled compound present in the original urine sample was then calculated from this specific activity and the amount of unlabeled carrier used.

The possible presence in the urine of 2-Me_2AF produced by methylation *in vivo* of 2-Me-C^{14}-AF was similarly investigated by carrier isotope dilution. After chromatography on alumina and nine recrystallizations from dilute ethanol, the specific activity of the compound decreased at each step and finally reached zero.

The excretion of the demethylation product of 2-MeAF, 2-AF, in the urine was determined by the use of colorimetric methods (11).

Determinations of Radioactivity—Tissues, feces, and other dry samples were converted to CO_2 by wet combustion (12) and counted as duplicate $BaCO_3$ plates. Liquid samples or dilute solutions were plated directly on glass disks; suitable correction factors to convert counts of direct plates to those obtained by combustion were determined on representative samples and applied to all direct plates. Counting was carried out in a windowless gas flow counter with a 48 per cent efficiency for C^{14}. Samples with a very low radioactivity were counted by a Robinson type windowless counter (13) which had a background counting rate of 3.8 to 4.0 c.p.m.

The *carcinogenic studies* were carried out in three experiments, each containing eighteen female Buffalo strain rats 2½ to 3½ months of age at the start of the experiment. The animals were housed individually in raised screen bottom cages and had access to a mixed diet of natural foodstuffs[1] and tap water at all times. The chemicals 2-MeAF, 2-Me_2AF, and 2-acetylaminofluorene (2-AAF) were incorporated into the diet at a level of 250 mg. per kilo of diet, or at 1.23, 1.20, and 1.12 mmoles per kilo of diet,

[1] The diet was composed of the following constituents in per cent by weight: commercial casein 3.00, skim milk powder 22.75, whole wheat (hard spring) meal 60.52, yeast 1.00, whole liver powder (Wilson) 1.00, NaCl 1.40, iron citrate 0.13, cod liver oil 2.00, corn oil 5.20, propylene glycol 3.00.

respectively. The 2-AAF-containing diet was intended to serve as a positive control. The rats were maintained on their respective diets until they developed visible tumors or until death appeared imminent, at which time a careful autopsy was performed. The tissues were fixed in Zenker's formol solution and subsequently stained by hematoxylin and eosin.

EXPERIMENTAL

Studies in Metabolism; Distribution of Radioactivity in Normal Animals—Rats on the control diet excreted 54 per cent of the carbon 14 from the administered 2-Me-C^{14}-AF in the respiratory carbon dioxide in the 72 hour experimental period (Table I). Of this, 46, 6, and 2 per cent were excreted on the 1st, 2nd, and 3rd day, respectively. The specific radioactivity of the barium carbonate derived from the expired carbon dioxide was highest in the 6 to 8 hour period and dropped off markedly after 24 hours. The urinary activity followed a similar course, a total of 22 per cent of the dose being eliminated in that manner. Radioactivity was found in all the organs analyzed.

Radioactivity was present in the choline and serine isolated from the carcass and the liver (Table II) and in the urinary creatinine. Degradation of the choline samples showed that 60 and 26 per cent of the C^{14} was located in the trimethylamine portion of carcass and liver choline, respectively. Degradation of the pure serine indicated that 98 to 103 per cent of the C^{14} was incorporated in the β position of this amino acid.

Solvent system A yielded the best separation of the complex mixture of urinary metabolites on the paper chromatograms. The autoradiographs showed intense spots with R_F values of 0.04, 0.12, 0.21, 0.25, and 0.49; other spots appeared at 0.30, 0.44, 0.64, 0.70, 0.82, 0.91, and 0.97. The last spot corresponded to that obtained with unchanged 2-Me-C^{14}-AF. Solvent system B or C gave a more reliable indication of the relative amounts of 2-Me-C^{14}-AF in comparative experiments since this compound alone moved near the solvent front; other metabolites had low R_F values or did not move at all. Thus, the unchanged compound appeared on the chromatograms of a urine sample collected in the first 24 hour period. However, only a trace of 2-Me-C^{14}-AF was present in urine obtained during the 2nd day.

Carrier experiments showed that only a minor amount, approximately 3 per cent of the urinary radioactivity collected on the 1st day, was due to 2-Me-C^{14}-AF. Similar carrier experiments with 2-dimethylaminofluorene gave a product counting within background rates which indicated that, if 2-MeAF is further methylated *in vivo*, the product is not excreted in the urine.

An aliquot of the urine collected on the 1st day contained 455,000 c.p.m. per ml., equivalent to 737 γ of 2-Me-C^{14}-AF. Colorimetric analysis for

diazotizable material gave the equivalent of 30 γ per ml. of free 2-AF, which thus accounted for 4.1 per cent of the urinary metabolites.[2]

TABLE I

Distribution of Radioactivity 72 Hours after Single Oral Dose of 2-Methyl-C^{14}-aminofluorene in Normal, Riboflavin-Deficient, and Pantothenic Acid-Deficient Rats

	Per cent of dose administered						
	Normal*			Riboflavin-deficient†			Pantothenic acid-deficient‡
			Average			Average	
Respiratory CO_2	55	53	54	30	31	31	52
Urines	19	24	22	44	46	45	21
Feces	12	12	12	4.2	3.9	4.1	7.9
Carcass	4.6	5.4	5.0	3.4	2.5	3.0	5.0
Gastrointestinal tract + contents§	2.4	4.0	3.2	4.5	2.9	3.7	3.1
Other organs‖	5.7	5.4	5.6	3.5	4.0	3.8	6.7

* The results with two animals administered 3.27 and 1.41 $\times$ 10^7 c.p.m., respectively.

† The results with two animals administered 3.03 and 1.21 $\times$ 10^7 c.p.m., respectively.

‡ Radioactivity of one deficient rat administered 2.54 $\times$ 10^7 c.p.m.

§ Stomach, small intestine, large intestine, cecum.

‖ Bladder, blood, brain, heart, kidney, liver, lungs, spleen.

TABLE II

Incorporation of Radioactivity from 2-Me-C^{14}-AF into Choline and Serine

	Normal rat		Riboflavin-deficient rat	
	Per cent of dose	Specific activity	Per cent of dose	Specific activity
Liver choline	0.13	580 (92)*	0.098	410 (93)*
Carcass choline	0.11	820 (61)*	0.035	330 (42)*
Liver serine†	0.53	200	0.200	110
Carcass serine†	0.83	20	0.77	16

Specific activity is stated in counts per minute per mg. of compound.

* Carrier choline hydrochloride was added after the isolation of choline reineckate prior to the recrystallization steps. The amount added is indicated in parentheses.

† 225 mg. of DL-serine carrier were added to 300 mg. of crude protein prior to hydrolysis.

Distribution of Radioactivity in Pantothenic Acid-Deficient Animals—The distribution and excretion data are given for only one pantothenic acid-

[2] Detailed colorimetric studies on the metabolism of 2-MeAF and 2-Me_2AF have recently been reported by Dyer (14).

deficient rat because it was difficult to secure survival of a rat for the required time of 72 hours after a dose of 2-MeAF in a severely deficient animal. The signs of the pantothenic acid deficiency appeared suddenly and the resistance of the treated animals decreased rapidly, so that death often occurred before the entire experiment could be concluded. However, the initial rate of radioactivity excreted in the urine and exhaled as CO_2, as well as the chromatograms of the urines collected in two partial runs, supports the results described for the single rat. 52 per cent of the radioactivity was exhaled in 72 hours, with 43, 6, and 3 per cent being found after 1, 2, and 3 days, respectively. The specific activity of the CO_2 was highest between 6 and 9 hours. The urine accounted for 21 per cent of the dose, and contained 14.6, 4.1, and 2.7 per cent, respectively, in the 3 successive days. The chromatograms of the urine showed the same number of spots with identical R_F values as those of the normal animals. Hence, this deficiency did not affect the metabolism of 2-MeAF.

Distribution of Radioactivity in Riboflavin-Deficient Rats—In this case also some difficulty was experienced in keeping the animals on the deficient diet alive for the 3 day experimental period after administration of the carcinogen. The results listed in Table I are averages obtained with two animals. Further data derived from three incomplete runs with other rats on a deficient diet support these results. In 1, 2, and 3 days, 23.5, 5, and 2.5 per cent respectively of the dose, or a total of 31 per cent, was expired as carbon dioxide, while the urines accounted for 37, 6, and 2 per cent, or a total of 45 per cent. A large portion of this radioactivity was due to excretion of unchanged 2-Me-C^{14}-AF, as indicated by the intense spot with an R_F value corresponding to this compound on the autoradiographs of the chromatograms in three solvent systems and by carrier isotope dilution experiments. In the first 24 hour period, 28 per cent of the urinary activity was associated with 2-MeAF, as compared to 3 per cent in normal rats.

Thus, the increased excretion of unchanged 2-MeAF, the lowered exhalation of radioactive carbon dioxide, the decreased activity (Table I) in the organs and carcass, and the appreciable decline in the specific and total activity of the serine and choline (Table II) indicated that riboflavin deficiency diminished the ability of the rat to demethylate the carcinogen.

Studies on Carcinogenicity—2-MeAF was fed for the entire experimental period averaging 11.7 months. 2-Me$_2$AF was administered for an average of 11.6 months, but the animals were continued on the control diet 3.5 months longer. The carcinogen intake (Table III), calculated from individual food consumption, varied because of differences in food intake and survival time. Survival was longer and there was a greater increase in weight for both groups receiving the methyl derivatives than for the animals fed 2-AAF.

Under our conditions, 2-MeAF and 2-Me_2AF produced multiple tumors at points distant from the portal of entry (Table IV). With 2-MeAF, 2-Me_2AF, and 2-AAF, fourteen of sixteen, fourteen of seventeen, and six-

TABLE III

Average Body Weight and Carcinogen Intake

Period of experiment in wks.	No. of rats surviving	Average body weight, gm.	Average daily intake, mg. per kilo body weight	Average total intake per rat cumulative period, mg.
2-MeAF				
1–40	13	195	12.2*	666
41–50	9	223	12.4	860
51–60	6	234	10.8	1038
61–75	1	249	9.6	1298
2-Me_2AF				
1–42	15	214	11.5*	748
43–54	13	236	10.3†	850
55–76	5	246	10.0†	1060
2-AAF				
1–33	17	187	12.1*	522
34–40	8	216	10.7	634
41–50	3	212	10.3	787

* Continuous ingestion.

† Alternate ingestion of 2-Me_2AF at weekly intervals. Intake values are based on the weeks in which 2-Me_2AF was ingested.

TABLE IV

Distribution and Frequency of Tumors Induced in Female Rats by Ingestion of 2-MeAF and 2-Me_2AF

Compound		Site and frequency of tumor formation*							
		Breast	Liver	Ear duct	Uterus	Stomach	Adrenal	Periotic area	Pituitary
2-MeAF	No. of tumors	18	8	6	1	1	1	1 (Sarcoma)	
	" " rats	9	8	5	1	1	1		
2-Me_2AF	" " tumors	15	9	2	2				2
	" " rats	11	9	2	2				2
2-AAF	" " tumors	25	12	5					
	" " rats	16	12	4					

* All the tumors except one mammary tumor were verified by microscopic examination of the tissues.

teen of sixteen rats were tumor-bearing. Bielschowsky and Bielschowsky (4) found no liver tumors in female rats in the experiments with the methyl derivatives, while only a low incidence of liver tumors was obtained in the male pie-bald rats of the Sheffield strain. In addition, Miller *et al.* (5) found no liver tumors in either male or female rats fed 2-Me_2AF for 8 months and kept on a control diet 2 months longer. The Buffalo strain rat used in our laboratory is somewhat resistant to the development of liver tumors by 2-AAF (15). Therefore, the induction of liver tumors by 2-MeAF and 2-Me_2AF in approximately 50 per cent of our female rats seems all the more significant.

DISCUSSION

The similarity in the rate of excretion and the total amount of radioactivity in the respiratory CO_2 of normal rats treated with the liver carcinogen 4-dimethyl-C^{14}-aminoazobenzene (3) and with 2-Me-C^{14}-AF are noteworthy, although of course this relationship might be coincidental. These two compounds are similar in structure in so far as the alkyl group is attached to an aromatic amino nitrogen. Furthermore, both compounds are carcinogenic to the liver, although 2-MeAF is not exclusively so. On the other hand, of the dealkylated products, 4-aminoazobenzene is carcinogenic only to the extent that methylation occurs (16), whereas 2-aminofluorene is a potent carcinogen which affects a number of tissues.

Our experiments show that further methylation of 2-MeAF appears somewhat unlikely as 2-Me_2AF was not excreted in the urine. This is in contrast to the easily reversible methylation of 4-methylaminoazobenzene to form 4-dimethylaminoazobenzene (17). Correspondingly, 2-MeAF is not present in the urine of rats fed 2-acetylaminofluorene-9-C^{14}, although 2-AF is obtained (18).

The occurrence of radioactivity in the β-carbon of liver and carcass serine, in the methyl and ethanolamine parts of choline, and in urinary creatinine suggests that the pathway of the methyl carbon of 2-MeAF leads to formaldehyde or related 1 carbon compounds (19) similar to that of the methyl group of 4-methylaminoazobenzene. However, there is a striking difference between the effect of a low riboflavin level on the demethylation of an azo dye, as described by Miller *et al.* (2), and our findings on the demethylation of 2-Me-C^{14}-AF. In our experiments the respiratory radioactivity and the activity incorporated in the serine and choline in deficient rats were considerably lower than in normal animals. In contrast, Miller *et al.* (2) found an increase in the radioactivity excreted in the CO_2 and incorporated into the serine with riboflavin-deficient rats fed 3′-methyl-4-dimethyl-C^{14}-aminoazobenzene.

Our results clearly indicate that the vitamin deficiency actually inhibits

he initial reaction in the sequence of steps from 2-MeAF to serine, choline, and carbon dioxide, since the chromatograms as well as the isotope dilution analysis demonstrated a considerable excretion of unaltered 2-MeAF in the urine of the animals on a deficient diet as compared to those on a normal diet. Thus, the enzyme system carrying out demethylation may be a flavoprotein, or the reaction could be indirectly coupled to a flavoprotein as suggested by La Du *et al.* (20). An earlier case of a demethylase containing flavin-adenine dinucleotide as a prosthetic group has been recorded by Moritani *et al.* (21) and Hirohata (22).

SUMMARY

1. The respiratory carbon dioxide, urine, and feces, collected over a 72 hour period, accounted for 54, 22, and 12 per cent, respectively, of the radioactivity from 2-methyl-C^{14}-aminofluorene administered to normal rats. Riboflavin deficiency altered this distribution to 31, 45, and 4 per cent, respectively, while pantothenic acid deficiency had no appreciable effect.

2. Liver and carcass serine and choline and urinary creatinine were radioactive. The specific radioactivity of serine and choline was lower in riboflavin-deficient than in normal animals.

3. Unchanged 2-Me-C^{14}-AF accounted for 3 and 28 per cent of the urinary radioactivity in normal and riboflavin-deficient rats, respectively.

4. Evaluation of the effect of dietary conditions on the demethylation of 2-Me-C^{14}-AF leads to the tentative conclusion that a flavin cofactor plays a rôle in this reaction.

5. 2-Dimethylaminofluorene, a potential metabolite of 2-Me-C^{14}-AF, was not present in the urine. A diazotizable amine amounted to about 4 per cent of the urinary metabolites excreted in 24 hours.

6. Multiple tumors, including hepatomas, were induced in Buffalo strain female rats by feeding 2-methylaminofluorene, 2-dimethylaminofluorene, and 2-acetylaminofluorene at a level of 0.250 gm. per kilo of diet over 7.5 to 11.5 months.

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PRECURSORS OF THE FOLIC ACID-ACTIVE FACTORS OF BLOOD*

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We have previously (1) shown the presence of relatively high folacin-like activity (for *Lactobacillus casei*) in dialysates obtained from human blood. It will now be shown that the active material, termed FS, is not present in blood as such, but results from the interaction of precursor factors which occur in blood cells and plasma. With the precursor material of the cells being termed FSP (FS precursor) and that of plasma PF (plasma factor), the present evidence can be schematized by the expression FSP + PF → FS. This expression is non-committal as to the multiplicity of any or all of these factors.

EXPERIMENTAL

Analytical Method—Freshly drawn human blood is diluted 750-fold with 0.05 M potassium phosphate buffer of pH 6.1 to which 0.05 per cent ascorbic acid has been added before use ("AP buffer"). After appropriate incubation (see below) proteins are coagulated by heating for 1 or 2 minutes at 120° in a steam autoclave. After centrifugation the supernatant fluid is used for bacterimetry with *L. casei* (3). If purified fractions are studied, these steps are suitably modified. The previously (3) described basal medium has been simplified by preparing it *in toto* as a dry mixture, aliquots of which are dissolved as needed. This has become possible by replacing the solution of enzymatic casein hydrolysate by a dry preparation of an acid hydrolysate of casein[1] and tryptophan. This medium is slightly less sensitive than the earlier one, and the standard levels used are 0.06, 0.12, and 0.18 μμmoles of folacin per ml. of finished medium.

Rôle of Ascorbic Acid—This analytical procedure has entirely replaced the earlier one, based on dialysis (1), because ascorbic acid proved to prevent loss of FS during heat coagulation as well as during storage. Pre-

* Blood folic acid studies II. For the first paper of this series, see Toennies *et al.* (1). Aided by a research grant (No. C-1479) from the National Cancer Institute of the United States Public Health Service and by an institutional research grant of the American Cancer Society. Presented in part at the Forty-fifth, 1954, and Forty-sixth, 1955, meetings of the American Society of Biological Chemists and at the Third International Congress of Biochemistry (2).

[1] Hycase, Sheffield Farms Company, New York.

sumably autoxidation is the main cause of the previously observed losses. Ascorbate cannot be replaced by cysteine, and it should be used at the specified level since higher concentrations of ascorbate interfere with protein coagulation.

FS Activity of Plasma and Cells—Washed blood cells and plasma, when assayed separately under conditions which yield high FS activity for whole blood, are nearly inactive. Combination of solutions of cells and plasma reestablishes the whole blood activity only in part, but full activity results when cells and plasma are recombined prior to dilution.

TABLE I

Contribution of Blood Components to FS

Cells and plasma were separated by centrifugation in silicone-coated tubes; L and H designate two different blood specimens of low and high FS level, respectively.

Blood	Cells	Plasma	FS value, μμmoles per ml. blood
L			36
	L		5
		L	0
	L	"	33
H			250
	H		42
		H	3
	H	"	280
	L	"	35
	H	L	280

By separating the bloods of persons with high and low FS values into cells and plasma and, without washing or dilution, recombining these in different ways, it could be shown that the inactive precursor of the cells (FSP) rather than the plasma factor (PF) determines the FS level of an individual blood. This is illustrated in Table I.

Rôle of Incubation—When dialysis as described before (1) is preceded by incubation of a blood hemolysate at pH 6.1, the FS value is greatly enhanced, as shown in Fig. 1. Since the results are similar when autoclaving in the presence of ascorbate is used instead of dialysis, 90 minutes of incubation at pH 6 and 38° prior to protein removal was adopted as standard procedure. As neither washed cells nor centrifuged plasma (or serum, see below) yields appreciable amounts of FS under these conditions, an interaction FSP + PF → FS must be assumed.

Partial Purification and Some Characteristics of FSP—A stabilized dry preparation of the cell precursor material can be obtained by the following

procedure: To 100 ml. of unwashed human red cells[2] add 100 ml. of 0.05 M NH_4OH for hemolysis. The pH of the hemolysate should be between 7.90 and 8.00; adjust if necessary. Add 800 ml. of 50 per cent (volume per volume) acetone to produce a 40 per cent acetone mixture. Remove and discard the resulting precipitate. Add acetone to the filtrate until its concentration is 85 per cent (volume per volume). Remove the new precipitate by filtration and centrifugation and lyophilize it. One obtains approximately 2.9 gm. of a brownish red powder (FSP precipitate) which

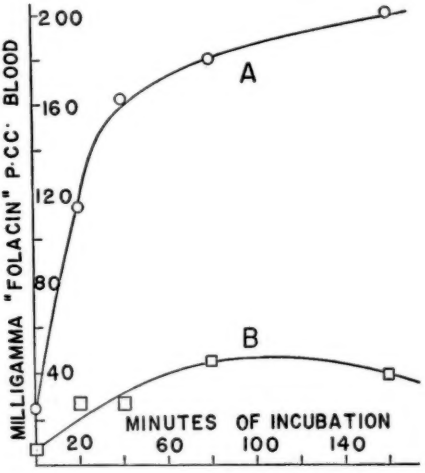

FIG. 1. Effect of incubation on FS value. Dilutions of the blood of two individuals (Curves A and B) with 0.05 M phosphate buffer of pH 6.1 were incubated at 38° and samples withdrawn at different periods were dialyzed against the same buffer for 18 hours at 2°. The activities found in the permeates are shown.

contains about 10 per cent of the original hemoglobin and 50 per cent of the original FSP. Fig. 2 illustrates the basis for this procedure, *i.e.* differential precipitation of FSP and hemoglobin. The product cannot be further purified by repetition of the same process, apparently because the solubility properties of the remaining hemoglobin have been altered by the solvent. In a specific example, red cells which contained 0.054 μμmole of FS and 0.66 μμmole of FSP per mg. of dry weight gave a 9 per cent yield of purified precipitate which contained per mg. 0.26 μμmole of FS and 4.8 μμmoles of FSP. The material is best dissolved in 0.04 M ascorbate buffer

[2] We wish to thank Mr. M. G. Coady of the plasma processing service of Sharp and Dohme for generous supplies of these cells.

of pH 3.8. Such a solution when incubated at 38° generates additional FS slowly without the interaction of plasma; in the above preparation the FS level increased from 0.26 to 1.12 and 1.74 μμmoles per mg. after 2 and 4 hours of incubation, respectively. Fresh washed cells also slowly liberate FS under similar conditions, while at pH 6 this does not occur with either

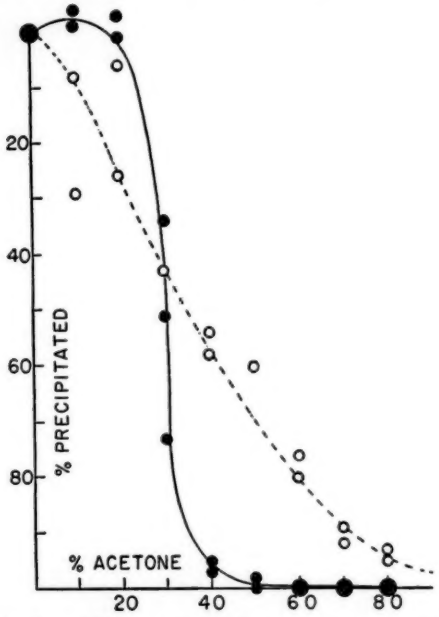

FIG. 2. Precipitation of FSP and hemoglobin by acetone. Aliquots of a mixture of equal volumes of red cells and 0.05 M NH_4OH, adjusted to pH 7.9 to 8.0, are further diluted with 9 volumes of different acetone-water mixtures. Determinations on the resulting supernatant solutions of hemoglobin (spectrophotometrically) and FSP (bacterimetrically from the difference in FS obtained on incubation with and without plasma) yielded the data shown: ●, hemoglobin; ○, FSP.

fresh cells or the FSP precipitate unless the plasma factor is present. Freezing and thawing also can liberate FS from cells, but the relevant conditions are not yet clearly defined. The FSP precipitate can be further purified by shaking it with water (20 ml. per gm.) at room temperature for 15 minutes and lyophilizing the filtrate. The resultant straw-colored dry product contains most of the FSP activity and only 10 to 20 per cent of the weight of the FSP precipitate.

FSP is thought to be a low molecular component of the non-hemoglobin intracellular proteins of the erythrocyte, because (*a*) it passes through a

cellophane membrane at a slow rate, *i.e.* approximately one-sixth as fast as FS, (*b*) red cell ghosts (prepared according to Ponder (4)) are found to have no FSP activity, and (*c*) a layer rich in white cells,[2] obtained by pooling of the buffy layers from human red cell separations, shows no increased FSP content, although it shows significant enhancement of *Leuconostoc citrovorum* activity. This would seem to rule out the white cells as the seat of FSP, since, as previously pointed out (1), the magnitude of the activity of whole blood dialysates for *L. citrovorum* is accounted for by

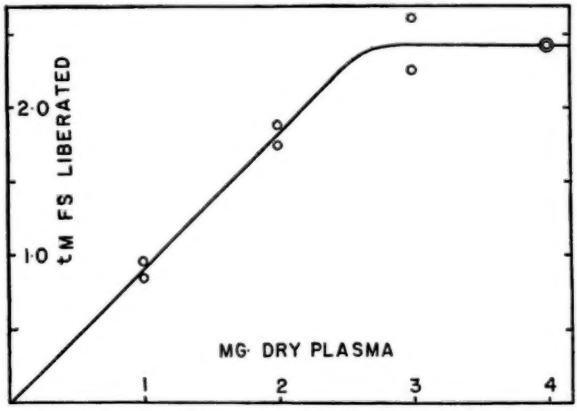

FIG. 3. Liberation of FS (*tM* = micromicromoles) from an FSP preparation by lyophilized plasma. To 100 μl. portions of a 1 per cent solution of FSP precipitate in ascorbate buffer at pH 3.8, 100 μl. portions of 1, 2, 3, and 4 per cent solutions of lyophilized plasma were added, followed by 10 ml. of AP buffer. After incubation, autoclaving, and centrifugation, FS was determined with 3 ml. of the supernatant fluid and 3 ml. of basal medium.

the leucocytes if their folic acid activity is entirely due to folinic acid, as shown by Swendseid *et al.* (5).

Interpretation—In the proposed scheme, FSP + PF → FS, we tentatively consider PF as "enzyme" and FSP as "substrate" (rather than vice versa), because according to the evidence presented (*a*) it is the level of FSP, rather than of PF, which determines the amount of FS yielded by an individual blood on incubation, and (*b*) under certain conditions FSP gives rise to FS without the action of plasma. Accordingly, a more specific formulation of the proposed working hypothesis would be thus FSP $\xrightarrow{PF}$ FS + ?.

Partial Characterization of PF—PF is measured by its capacity to liberate FS from FSP on incubation at pH 6. The pattern of this reaction is illustrated by Fig. 3. By comparisons in which plasma or serum was

used in suboptimal proportions, it could be shown that (*a*) if fresh plasma is allowed to coagulate, the resulting serum retains the PF activity, (*b*) serum kept in the refrigerator for 1 year loses little or none of its PF activity, (*c*) on dilution of serum or plasma PF activity diminishes, at rates which tend to increase with increasing dilution, and (*d*) commercial preparations of lyophilized citrated human plasma are good sources of PF and

TABLE II

PF Activity of Plasma Fractions

Cohn Fraction No.	Specimen	Relative FS activity*	Approximate contribution of fraction to weight of whole plasma	Approximate total FS activity of fraction
		per cent	*per cent*	*per cent*
I	A	0	4–8	0
I	B	0	4–8	0
I	C	15	4–8	1
II + III	A	0	20–25	0
II + III	B	32	20–25	8
II + III	D	3	20–25	1
IV + V	A	163	70	114
IV-1	D	0	6	0
IV-4	C	136	12	16
IV-4	D	136	12	16
V	C	72	50	36
V	D	6	50	3
V	E	37	50	18
VI	A	0	2	0

Specimen A from K. Schmid in 1951; Specimen B from K. Schmid in 1955, "acid-citrate-dextrose, totally soluble;" Specimen C from K. Schmid in 1955, "made December 17, 1953;" Specimen D from R. B. Pennell in 1955; Specimen E from K. Schmid in 1955, "acid-citrate-dextrose, contains Zn."

* Compared with dry whole plasma as 100.

retain much of their activity even when stored for several years in daylight and at room temperature.

As to the seat of PF among the components of plasma, Table II presents the results of comparisons of the PF activity of a series of protein fractions obtained from the Harvard plasma fractionation laboratories.[3] Fraction IV + V, the apparent seat of PF, consists chiefly of serum albumin, but also contains specifically glyco-, lipo-, muco-, and other special proteins. Further investigation of Subfraction IV-4 seems indicated.

Sensitivity of PF and FS to Ultraviolet Light and Heat—In addition to

[3] We wish to thank Dr. K. Schmid, Dr. R. B. Pennell, and Dr. J. L. Oncley for supplying materials and information.

PF, crude plasma contains a certain amount of free FS. Measurements of plasma activity in the presence and absence of FSP, after dried plasma spots on paper had been exposed to heat or ultraviolet light, showed that FS activity is slightly impaired by heat and much more by ultraviolet light, while in the case of PF the effects are reversed: heat is more damaging than ultraviolet light. These observations accord with expectations if FS consists of low molecular compounds similar to folacin (6) and if PF is of protein nature, as is also suggested by its presence in highly purified plasma protein fractions.

Interaction of PF and FSP—While it was recognized early that dilution effects are important in the reaction FSP + PF → FS, their clear definition proved to be difficult. Systematic study was facilitated when the stable

TABLE III

Rôle of Combining Concentrations in Formation of FS from FSP and PF

Each mixture contained 100 μl. of plasma and 5 mg. of FSP precipitate. The results are in terms of micromicromoles of FS liberated per ml. of the blood represented by the FSP precipitate.

Concentration of plasma, per cent	Concentration of FSP ppt.			
	0.093 per cent	0.28 per cent	0.83 per cent	2.5 per cent
100	23	19	17	13
33	16	14	14	9
11	11	9	8	9
3.7	8	6	8	5

acetone precipitate could be used as a source of FSP instead of the variable and unstable fresh red cells.

The following experiment, which was preceded by many other exploratory ones, reveals the nature of the trends. Fresh liquid plasma as a source of PF and a 2.5 per cent solution of the FSP precipitate in 0.04 M ascorbate buffer of pH 3.8 were each diluted 3-fold in three successive steps, so that the resulting relative concentrations were $1:\frac{1}{3}:\frac{1}{9}:\frac{1}{27}$ with regard to PF as well as FSP. Aliquots containing equal quantities of PF (equivalent to 0.1 ml. of plasma) at different concentrations were combined with aliquots containing equal quantities of FSP (5 mg. of FSP precipitate) at different concentrations. After mixing, all combinations were brought to the same volume, incubated, and assayed as usual, including appropriate controls. AP buffer served as the diluent throughout. The net results, in terms of micromicromoles of FS liberated per ml. of the blood represented by the FSP concentrate, are listed in Table III which also shows the scheme of the experiment. It is apparent that the reaction is favored by high concentra-

tion of PF and at the same time by high dilution of FSP. It should be noted that the individual combinations differ only in regard to the concentration of the components at the time of mixing, since everything was equalized prior to incubation.

In the preceding experiment the ratio of reactants was 20 μl. of plasma per mg. of FSP precipitate. Table IV shows the summarized results of experiments in which incubation time and PF:FSP ratio were varied. Undiluted plasma and a 0.04 per cent solution of FSP precipitate were combined in different proportions. This experiment, together with the preceding ones, yielded the conclusion that for purposes of measuring total FSP potency the latter should be employed as dilute as possible, while

TABLE IV

Rôle of Incubation Time and PF:FSP Ratio in Yield of FS

The results are in terms of micromicromoles of FS liberated per ml. of the blood represented by the FSP precipitate.

Plasma per mg. FSP ppt.	Incubation time			
	0 min.	30 min.	60 min.	120 min.
μl.				
33	21	35	39	40
66	20	40	42	39
100	20	38	42	43
133	20	40	42	43

plasma should be used undiluted and in a ratio of 50:100 μl. per mg. of the present FSP preparation (or equivalent).

In order to measure PF activity, the material to be assayed should be employed as concentrated as possible and should be compared with fresh plasma at levels of 0 to 40 μl. per mg. of FSP precipitate. Incubation at 38° should be for 60 to 90 minutes.

DISCUSSION

If blood cells contain bound FS (FSP) and plasma contains enzymatic means for releasing it (PF), the question arises whether physiologically the two have a direct functional relationship. In terms of a working concept FSP may be viewed as a storage form of folic acid-active compounds which is sequestered in the red cell and becomes accessible to the action of PF as the red cell undergoes destruction at the end of its average life span of 120 days (7). This would account for the small amount of folic acid activity which we and others have found to be present in plasma (1). Hoarding of folic acid has been demonstrated for cells of *L. casei* (8), and

in the case of biotin similar observations have been made (9). It remains to be seen whether in the higher organism the erythrocyte subserves a similar hoarding function for folic acid-like factors, and perhaps other vitamins, and whether plasma is equipped to split other vitamin conjugates. The existence of plasma enzymes (conjugases) acting upon folic acid factors has been shown before (10, 11), but precursors contained in the blood cells appear not to have been previously observed.

SUMMARY

It is shown that the folic acid activity of blood hemolysates is the result of interaction between separate factors contained in cells and plasma. Evidence suggesting that the cells contain a precursor (or precursors) and the plasma an enzymatic factor (or factors) is presented, and partial purification and some characteristics of these are described. A simplified *Lactobacillus casei* assay procedure for FS (the active end-product), involving a dry mixture for the growth medium and the stabilization of FS by ascorbate, is also presented.

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MULTIPLICITY OF THE FOLIC ACID-ACTIVE FACTORS OF BLOOD*

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(Received for publication, December 15, 1955)

Previous work in this laboratory (2) has shown that under proper conditions human blood yields much higher folic acid activity values than those found by prior authors. Microbiological responses indicated more than one factor to be involved. According to the evidence, neither folacin (pteroylglutamic acid), nor pteroyltriglutamic acid, nor folinic acid seemed to be a major component of FS (the designation adopted for the active material). It was also shown (3) that FS is not present as such in blood but results from the interaction, during and after hemolysis, of factors contained in erythrocytes and plasma.

The preceding report (3) deals with a study of the precursors, and the present one discusses the end-products (FS). The preparation of stable concentrates of FS and their fractionation will be described. It will be shown that six or more folic acid-like compounds appear to be involved, and their relation to known factors will be considered.

EXPERIMENTAL

Analytical—As previously described (3), FS activities are measured with *Lactobacillus casei* and the results are expressed in micromicromoles. The same system is used to measure and express *Streptococcus faecalis* and *Leuconostoc citrovorum* activities.

Starting Material—In the commercial production of lyophilized human plasma large amounts of unwashed human red cells are obtained as a by-product. Because such cells are readily available and yield substantial amounts of FS, they have been employed in the present purification studies.[1] They are frozen and thawed prior to hemolysis in order to increase the yield of FS (3).

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[1] We acknowledge with appreciation the help of Mr. M. G. Coady of Sharp and Dohme, Division of Merck and Company, Inc., Glenolden, Pennsylvania, in supplying the red cells.

Deproteinization—Heat coagulation of blood proteins leads to loss of FS unless ascorbic acid is present (3). In the preparation of the concentrate used in most of the present purification studies, heat coagulation in the presence of ascorbic acid represented the first step. Alternative procedures have been explored since, such as protein precipitation by trichloroacetic acid, coprecipitation with a lignin derivative (Indulin A[2]), or dialysis. All of these methods, and particularly the last, result in a cleaner product than heat coagulation.

Pilot Plant Concentrate[3]—20 liters of frozen and thawed red cells were added to a solution of 2.4 kilos of ascorbic acid in 480 liters of 0.05 M potassium phosphate buffer of pH 6.1. The solution was heated with stirring to 95° until a supernatant sample was colorless. The coagulated protein was removed by filtration and 200 gm. of Norit A were added to the filtrate. After stirring for 1 hour the charcoal was filtered. Celite was used as a filter aid. FS was desorbed by agitating the charcoal with a total of 6 liters of ammoniacal aqueous ethanol (10, 40, and 50 per cent by volume of concentrated NH_4OH, H_2O, and 95 per cent C_2H_5OH, respectively) and the filtrate was evaporated *in vacuo* and lyophilized after removal of insoluble material. Assays showed that this product (19.9 gm.) contained 5.9 μμmoles of FS per mg. compared with 0.09 μμmole per mg. of dry red cells. Thus purification is nearly 70-fold, while the absolute yield is 9 per cent.

Stability—In the presence of ascorbate FS is stable for long periods in phosphate, pH 6, at 2°, while in the absence of ascorbate activity is completely lost in 2 weeks (2). Acid solutions and, more slowly, alkaline solutions destroy FS. Dry preparations have shown no change in activity after storage for 6 months at room temperature.

It was noted previously (2) that on storage of FS the ratio of *S. faecalis* activity to *L. casei* activity undergoes a marked increase. This also occurs during the purification procedure: the ratio increased from 0.1 in the deproteinized solution to 0.5 after charcoal adsorption and desorption with alcoholic ammonia. The activity ratio, *L. citrovorum* to *L. casei*, remained approximately 0.03. Enhancement of *S. faecalis* activity through the action of alcoholic ammonia was also noted by Hakala and Welch (4); in their case it was attributed to degradation of the triglutamate form of folinic acid to the diglutamate.

Solubility—FS appears to be soluble in water, aqueous methanol, and ethanol at neutral pH, but insoluble in the water-immiscible organic solvents. At low pH, FS could be distributed between water and *n*-butanol.

[2] West Virginia Pulp and Paper Company, Charleston, South Carolina.

[3] The pilot plant operations were carried out at the Sharp and Dohme plant in West Point, Pennsylvania, under the guidance of Dr. Lemuel D. Wright. We are indebted to him and other officials of the company.

Fractionation—Separation of FS activity into several components has been obtained by elution from basic resins, by solvent partition, by paper electrophoresis, and by paper chromatography. The last technique has yielded the most detailed results. The locations of microbiologically active

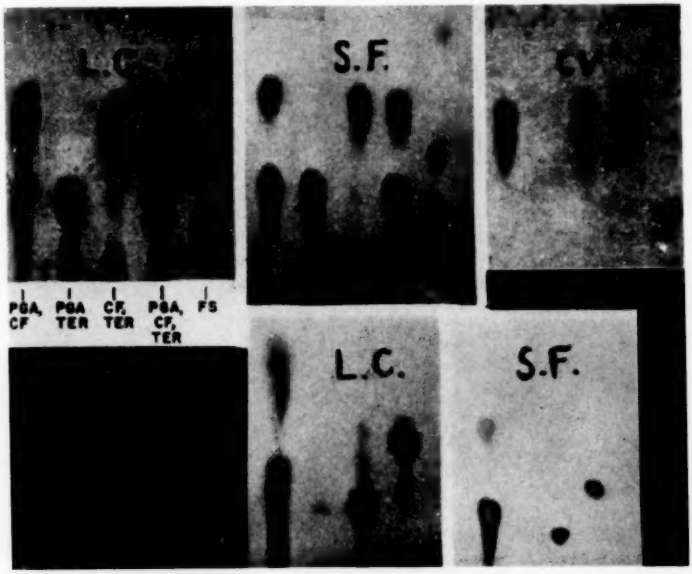

FIG. 1. Paper chromatography of FS and reference substances. The upper three panels show tetrazolium bioautography of identically developed sheets on agar seeded with *L. casei*, *S. faecalis*, and *L. citrovorum*, respectively. Combinations of PGA, TER, and CF were used for comparison, as indicated. The sheets were developed (ascending) with a 52:28:20:0.3 combination of *n*-butanol, water, ethanol, and acetic acid. The lower two panels show (from left to right) PGA + TER + CF and Subfractions 1, 2, and 3 of FS (see the text). Schleicher and Schuell 589 green ribbon paper was used. *L. C.* = *L. casei*, *S. F.* = *S. faecalis*, and *CV* = *citrovorum*.

substances[4] on paper were made visible by the technique of tetrazolium bioautography (5), in which a modified medium was employed (3).

The results presented in Fig. 1 show (upper three panels) that in the *n*-butanol system FS is resolved into two major (FS-1 and FS-2) and one minor (FS-3) factors, and that none of these is identical with pteroylglutamic acid (PGA), teropterin (TER), or citrovorum factor (CF). The minor factor, FS-3, shows only *S. faecalis* activity, and only FS-1 shows *L. citrovorum* activity. For purposes of fractionation crude FS concentrate was

[4] We are indebted to Dr. T. H. Jukes (Lederle Laboratories Division, American Cyanamid Company) for samples of the rarer compounds.

applied to a large sheet in a transverse line and developed with the *n*-butanol system. Three consecutive horizontal strips, representing the zone below the first FS spot and the zones of the two main FS spots, were cut off and eluted with water by a procedure involving evaporative concentration (6). The three concentrates were rechromatographed side by side as individual spots, together with the control mixture of PGA, TER, and CF, and the resulting sheets were bioautographed with *L. casei* and *S. faecalis*. The lower two panels of Fig. 1 show the result. It is apparent that the two

FIG. 2. Paper chromatography of FS and reference substances; development with a 5 per cent citric acid solution brought to pH 9 with ammonia and saturated with isoamyl alcohol (7); tetrazolium bioautography with *L. casei*. The spots applied contained (from left to right) PGA, CF, TER, a combination of these, FS, N^{10}-formylpteroylglutamic acid, anhydroleucovorin, and pteroyl-α-glutamylglutamic acid.

major FS subfractions have been well separated by the strip method and that their R_F values, relative to the controls, have not been changed in the process.

Resolution of FS into two major components also occurred when 5 per cent Na_2HPO_4 saturated with benzyl alcohol (7) was used for chromatography. While folacin, teropterin, and folinic acid gave R_F values of 0.49, 0.77, and 0.68, respectively, FS-1 appeared at 0.92 and FS-2 at 0.86. Inversion of the relative positions of the two was established by evaporative elution of the fractions and rechromatography in the *n*-butanol system. A higher degree of resolution was achieved by the citrate-amyl alcohol system of Wieland, Hutchings, and Williams (7). Fig. 2 shows that FS

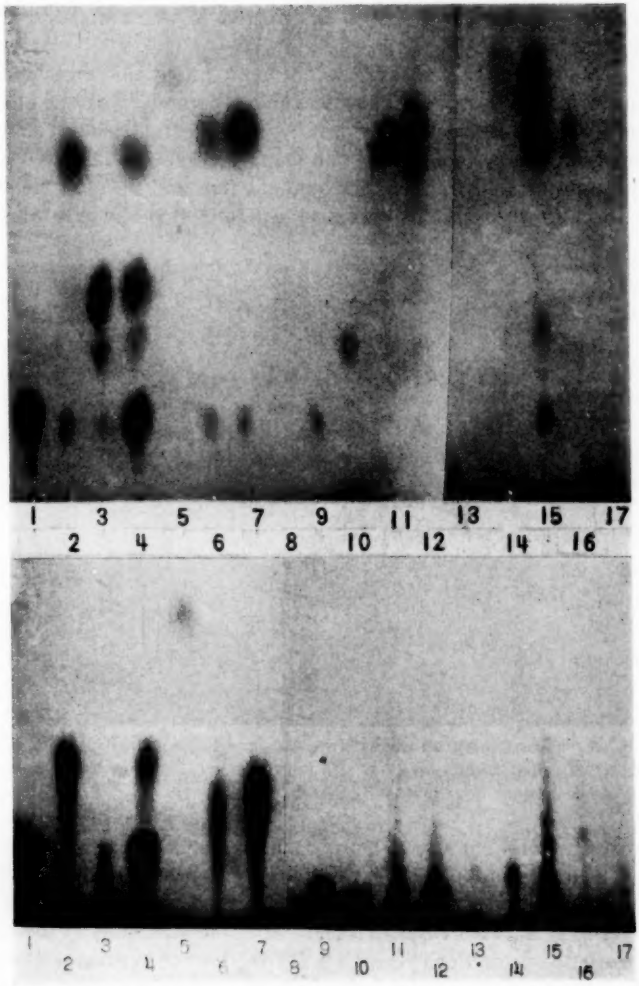

FIG. 3. Paper chromatograms of FS fractions and reference substances, bioautographed with *L. casei*. The numbers indicate spots of PGA (*1*), CF (*2*), TER (*3*), PGA + CF + TER (*4*), thymidine (*5*), anhydroleucovorin (*6*), formylpteroylglutamic acid (*7*); citrate fractions: *A* (forefraction, *8*), *B* (*9*), *C* (*10*), *D* (*11*), *E* (*12*), *F* (after fraction, *13*); *n*-butanol fractions: *a* (forefraction, *14*), *b* (*15*), *c* (*16*), *d* (after fraction, *17*). The upper picture shows the result of chromatography with the citrate solvent; the lower one, with the *n*-butanol solvent.

forms at least four distinct zones, the highest of which appears to be of multiple composition.

FS was now fractionated, by strip chromatography and cross-elution of sections, with the citrate solvent system and, separately, with the *n*-butanol system. The citrate system yielded the four fractions shown in Fig. 2, and the *n*-butanol system yielded two main fractions as illustrated by Fig. 1. The concentrated fractions, including a fore and an after fraction in each case, were then chromatographed separately side by side with some

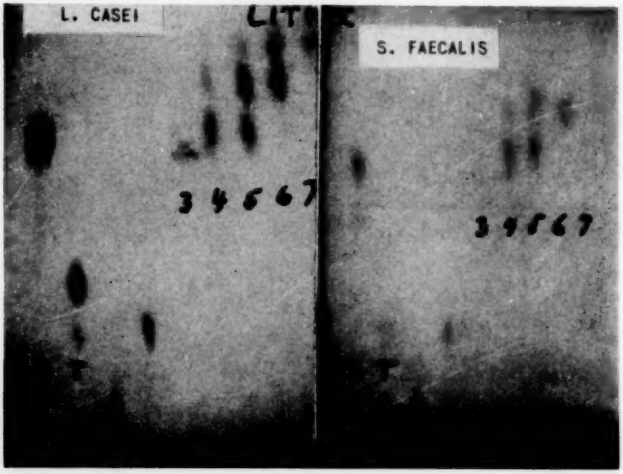

FIG. 4. Paper chromatograms of FS fractions and reference substances, bioautographed with *L. casei* and *S. faecalis*. FS fractions 1 to 7 were obtained by eluting cuts of a chromatopack developed with citrate solvent.

reference substances, by using the *n*-butanol solvent for one sheet and the citrate solvent for another. The results are shown in Fig. 3. The citrate run of the citrate fractions (upper picture) confirms the existence of five or six FS components (see Nos. 9, 10, 11, 12), and according to the citrate run of the butanol fractions FS-1 (No. 15) has four or five components and FS-2 (No. 16) has one. On the basis of the R_F values in the citrate system four of the FS components could be identical with PGA, with the secondary spot of TER, with CF, and with anhydroleucovorin or formylpteroylglutamic acid. However, when the same fractions and reference substances are compared by *n*-butanol chromatography (lower picture), it becomes apparent that actually there is no identity between the reference compounds and the FS components. Other comparisons showed that in

the *n*-butanol system thymine resembles thymidine, while in the citrate system it runs about 15 per cent slower.

Fractionations on an increased scale were carried out with multiple paper sheets (chromatopile (8), chromatopack (9)), and Fig. 4 illustrates the results obtained. At least five discrete components are in evidence and differences among them with regard to effectiveness for the two test organisms are apparent.

DISCUSSION

Numerous recent studies indicate that the family of naturally occurring folic acid compounds consists of many structural variants. Many investigators postulate the existence of conjugates of pteroylglutamic acid, tetrahydropteroylglutamic acid, and folinic acid. In Sweden, Ericson has identified several of the folic acid-like factors present in yeast, algae, etc. (10), and he and his associates have also shown that there are at least nine different compounds, occurring in either free or bound form, which possess folic or citrovorum activity (11). In the present work pteroylglutamic acid, folinic acid, pteroyltriglutamic acid, N^{10}-formylpteroylglutamic acid, anhydroleucovorin, pteroyl-α-glutamylglutamic acid, thymine, and thymidine seem to be ruled out as major components of FS on the basis of direct comparisons. The fact that all FS components give good *L. casei* response argues against pteroic acid or its derivatives. Comparisons between reported R_F values and those observed for FS leave the possibility open that FS may contain pteroylglutamyl-γ-glutamic acid and the di- or triglutamates of folinic acid.

We wish to acknowledge with appreciation the technical assistance of Miss Patricia J. Raff and Miss Louise B. Kirschner.

SUMMARY

The folic acid-active material occurring in blood hemolysates has been concentrated by adsorption on charcoal after removal of blood proteins, followed by desorption from the charcoal with ammoniacal alcohol. The concentrate has been fractionated by various paper chromatographic procedures, with tetrazolium bioautography serving as a means of detection, and it has been shown that FS (the active material) has at least six components, and that none of these appears to be identical with the folic acid-active substances which at present are chemically well defined. All factors are characterized by high *Lactobacillus casei* activity, while their *Streptococcus faecalis* activity, and even more so their *Leuconostoc citrovorum* activity, is small.

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BIOSYNTHESIS OF RIBOSE IN ESCHERICHIA COLI GROWN ON C^{14}-LABELED GLUCOSE*

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The biosynthesis of ribose in *Escherichia coli*, strain B, has been studied by using the tracer techniques previously employed in an investigation of this synthesis in the chick (1). These procedures were designed to evaluate the importance, *in vivo*, of formation of ribose by direct conversion from hexose by loss of either end carbon (*cf.* (2–4)). Whether carbon 6 was lost as suggested by the "uronic acid-decarboxylation" hypothesis (*cf.* (5)), or carbon 1 was removed as would occur by the hexose monophosphate oxidative pathway, in a tracer experiment the distribution of C^{14} in the pentose would be expected to be similar to that of the remaining 5 consecutive carbons in the hexose. Dissimilar patterns would indicate the probable involvement of one or more other pathways. In the chick experiments (1), the patterns of C^{14} in the glycogen (representing the hexose pool) and in the ribose (from ribonucleic acid) were different, and it was concluded that the major pathway for the synthesis of ribose under these conditions was not directly from hexose by loss of either carbon 1 or carbon 6. Synthesis by combination of $C_3 + C_2$ units was suggested as being in accord with the observed results. One possible mechanism for such a process might be the condensation of glyceraldehyde phosphate and an active C_2 unit catalyzed by transketolase (6, 7).

A similar study in *E. coli*, however, has led to data which have been interpreted as confirming the conclusion of Lanning and Cohen (8): that most of the ribose in the ribonucleic acid of this organism was synthesized from hexose by loss of carbon 1.

Methods

Growth and Harvesting of Bacteria—E. coli, strain B, was grown with vigorous shaking[1] for 18 hours at 37° in a medium of inorganic salts and

* A preliminary report on this investigation was presented at the 127th meeting of the American Chemical Society, Cincinnati, Ohio, April, 1955.

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[1] 120 strokes per minute on a reciprocal shaker, model V, manufactured by the New Brunswick Scientific Company, New Brunswick, New Jersey.

C^{14}-labeled glucose as the sole carbon source. The SM8 medium of Spizizen *et al.* (9), with the concentrations of all constituents except glucose doubled, was used. The final concentration of glucose was 0.4 per cent. 1 liter of medium was inoculated with the cells grown during the previous 24 hours on an agar slant[2] and washed in with 5 ml. of sterile H_2O. The cells were harvested by centrifugation and washed twice with 25 volumes (25 ml. per gm. of cells wet weight) of 0.01 per cent unlabeled glucose and two times with 25 volumes of distilled H_2O.

Isolation of Ribose—The washed cells were suspended in 5 volumes of H_2O, and the suspension was added with stirring to 45 volumes of cold (−10°) acetone. After standing overnight at 2°, the insoluble residue was removed by centrifugation at 0°, suspended in 10 volumes of cold acetone, and again centrifuged. After the addition of Hyflo Super-Cel (0.2 gm. per gm. of original weight of cells), the residue was stirred with 20 volumes of 7 per cent trichloroacetic acid at 0° for 20 minutes, filtered, and washed with cold H_2O, alcohol, and ether (1). Ribose was isolated and purified from the acid-insoluble fraction (by using NaOH for the ribonucleic acid-deoxyribonucleic acid separation) as previously described (1). Unlabeled ribose was added as a carrier after the purification.

Degradation of Ribose—The distribution of C^{14} in the ribose was determined by fermentation with *Lactobacillus pentosus* (10).

Source of C^{14}-Labeled Glucose—Glucose-3,4-C^{14} was obtained from the glycogen of rat liver after administration of $NaHC^{14}O_3$. The distribution of C^{14} was determined as described by Bernstein *et al.* (11). Glucose-1-C^{14}, glucose-2-C^{14}, and glucose-6-C^{14} were obtained from the National Bureau of Standards.

Determination of Radioactivity—Radioactivity was determined in a gas phase counter (12) to a standard counting error of 3 per cent or less. All total carbon analyses were done by the method of Van Slyke and Folch (13).

Results

Table I presents the radioactivity data for the various experiments. When glucose-3,4-C^{14} was used as the carbon source, the ratio of carbons 2 to 3 in the ribose was 0.85, while the level of C^{14} in carbon 3 of the hexose was 94 per cent of that in carbon 4. The pattern of C^{14} in the ribose was qualitatively similar to that of carbons 2 to 6 of the glucose. The ribose obtained in the case of glucose-1-C^{14} had levels in carbons 1 and 5 of 14 and 25 per cent of that in the labeled carbon of the glucose. Values of 17 and 28 were found in carbons 2 and 4 of the pentose relative to 100 in

[2] The slants were grown in 20 × 150 mm. test-tubes at 37° for 24 hours in a medium consisting of 0.8 per cent Difco nutrient broth, 0.5 per cent NaCl, and 2 per cent agar.

carbon 2 of the glucose in Experiment 3. In addition, carbon 1 of ribose was diluted to 76 per cent of that of the labeled carbon in the glucose in this experiment. In the experiment in which glucose-6-C^{14} was used, carbon 5 of the ribose had only 74 per cent, while carbon 1 had 8 per cent of the activity in carbon 6 of the hexose.

TABLE I

C^{14} Distribution in Ribose Isolated from E. coli Grown on Labeled Glucose

For details of the procedures see the text.

Experiment No.	Carbon source	Relative specific activity (R. S. A.)*											R. S. A. of 100 equals‡	Ribose specific activity†‡	
		Glucose						Ribose†						Calculated§	Found‖
		C-6	C-5	C-4	C-3	C-2	C-1	C-5	C-4	C-3	C-2	C-1			
														$\times 10^3$	$\times 10^3$
1	Glucose-3,4-C^{14}	0.3	1.9	100	94	2.5	1.0	1.5	5.7	101	85	5.7	2.53 $\times 10^5$	1.01	1.07
2	Glucose-1-C^{14}	0	0	0	0	0	100	25	2.0	1.2	1.7	14	3.38 $\times 10^6$	2.97	3.00
3	Glucose-2-C^{14}	0	0	0	0	100	0	3.2	28	3.2	17	76	6.94 $\times 10^5$	1.77	1.81
4	Glucose-6-C^{14}	100	0	0	0	0	0	74	4.6	2.6	3.4	7.9	2.05 $\times 10^6$	3.79	3.99

* R. S. A. = (specific activity for the carbon in question × 100)/(specific activity of the glucose carbon given a value of 100).

† The values for C^{14} in ribose have been corrected for dilutions by carrier. Carrier dilutions were 130-fold in Experiment 1, 47-fold in Experiment 2, 52-fold in Experiment 3, and 96-fold in Experiment 4.

‡ The data are given in counts per minute per millimole of carbon.

§ (Average of R. S. A. for the 5 carbons × specific activity corresponding to an R. S. A. of 100)/100.

‖ By total combustion.

DISCUSSION

The data of Experiment 1 (glucose-3,4-C^{14}), in which the distribution of isotope in the pentose paralleled that of carbons 2 to 6 of the glucose, suggest that the major pathway for biosynthesis of ribose in *E. coli* is by loss of carbon 1 of hexose, as previously reported by Lanning and Cohen (8). These results further confirm the conclusion of Cohen (14), that the postulated "uronic acid-decarboxylation" pathway plays no significant rôle in the synthesis of ribose in this organism.

If ribose does arise directly by loss of carbon 1 from hexose, the pentose would be expected to be unlabeled in cells grown on glucose-1-C^{14}. When

they carried out such an experiment, Lanning and Cohen (8) actually found that the ratio of counts per minute per micromole in ribose to that in the glucose-1-C^{14} was 0.2:0.3 and concluded that a mechanism other than a direct conversion from hexose was also operating to a significant extent. Determination of the pattern of C^{14} in the ribose in Experiment 2 (glucose-1-C^{14}) showing that two-thirds of the total activity in the molecule was in carbon 5 and the remainder in carbon 1 suggests, however, that the direct pathway accounts for even more of the synthesis than supposed by Lanning and Cohen. The activity of carbon 5 in this experiment could be a consequence of randomization of C^{14} in the hexose phosphate pool prior to synthesis of pentose. A reversible conversion of hexose monophosphate to the triose phosphates and the equilibration of the latter catalyzed by the isomerase would result in such a randomization. The activity of carbon 4 in Experiment 3 (glucose-2-C^{14}) and the reduced specific activities of carbon 1 in the same experiment and of carbon 5 in Experiment 4 (glucose-6-C^{14}) would also be explained by this hypothesis. Isolation and degradation of a sample from the "hexose phosphate" pool should clarify this point.

Nevertheless, such randomization of the C^{14} in the hexose phosphate pool and direct conversion of carbons 2 to 6 of the hexose to ribose do not entirely explain the patterns of radioactivity found in the pentose. The radioactivities in carbon 1 of the ribose in Experiment 2 and in carbon 2 of Experiment 3 are cases in point. Synthesis of pentose by the "transketolase" mechanism (6, 7) in which the C_2 fragment, coming from carbons 1 and 2 of fructose-6-phosphate (15), would be condensed with a C_3 unit such as glyceraldehyde phosphate could account for these activities. By this process, carbons 1 and 2 of the hexose phosphate would become carbons 1 and 2, in the same order, of the ribose. Thus, in Experiment 2, some of the C^{14} from carbon 1 of the glucose-1-C^{14} would be found in carbon 1 of the ribose, and some of the radioactive isotope from the glucose-2-C^{14} would appear in carbon 2 of the pentose in Experiment 3. This mechanism could also be the reason for the decrease in the activity of carbon 2 of the ribose relative to carbon 3 of the glucose in Experiment 1, since in this case unlabeled carbon 2 of the hexose would contribute to carbon 2 of the pentose.

Presently available evidence suggests that the biosynthesis of ribose occurs by *at least* two pathways and that different organisms probably utilize these mechanisms to varying degrees. In the chick, the pentose appears to be synthesized via combination of smaller units, while in *E. coli* the synthesis would seem to proceed predominantly directly from hexose by loss of carbon 1. *Torula utilis* is reported (16) to utilize the direct pathway entirely, whereas rat liver appears to use both about equally (17).

It is of interest to note that glucose was the labeled substrate in those experiments favoring the direct pathway, while labeled precursors having

only 1 or 2 carbons were used in the cases in which another pathway was shown to be important. Experiments are in progress to determine whether, in *E. coli* grown on unlabeled glucose as the major carbon source and trace amounts of acetate-1-C^{14}, the data would still indicate the paramount importance of the direct pathway in the synthesis of ribose. In this connection, Plaut and Broberg (18) found C^{14} in carbons 1 and 5 of the side chain of riboflavin synthesized by *Ashbya gossypii* grown on glucose-1-C^{14} and preliminary data[3] indicate that, in riboflavin isolated from a culture of *Candida flareri* grown on glucose-3,4-C^{14}, the pattern of C^{14} in the ribityl moiety was not similar to any 5 consecutive carbons of the labeled glucose. In the synthesis of this "ribose"-like polyhydroxy chain, the data suggest a pathway other than a direct conversion from hexose, although glucose was the labeled substrate.

The author sincerely appreciates the interest of Dr. Arthur C. Curtis in the continuation of this work.

SUMMARY

1. Ribose from ribonucleic acid has been isolated from *Escherichia coli*, strain B, grown on glucose-3,4-C^{14}, glucose-1-C^{14}, glucose-2-C^{14}, and glucose-6-C^{14}. The patterns of C^{14} in the ribose were then compared with those in the glucose used as the carbon source. Similar patterns in the pentose and hexose were assumed to indicate synthesis of the pentose directly from hexose by loss of an end carbon; dissimilar patterns, synthesis by some other mechanism, possibly involving combination of small carbon units.

2. The patterns of C^{14} in the ribose and glucose were qualitatively similar, and the data were interpreted to indicate that ribose was synthesized in *E. coli* predominantly from hexose by loss of carbon 1.

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BIOCHEMISTRY OF THE SPHINGOLIPIDES

IX. CONFIGURATION OF CEREBROSIDES*

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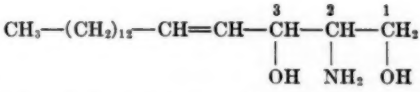
The chemical structure of sphingosine was established by Carter *et al.* (1) as follows:



Subsequent stereochemical studies show that the double bond has the trans configuration (2, 3) and that the amino carbon atom has the D configuration (4, 5). Several investigators (5–7) have presented evidence for the *erythro* configuration of the 2nd and 3rd carbon atoms of natural dihydrosphingosine. Thus it is now conclusively established that sphingosine, as isolated by hydrolysis of cerebrosides in anhydrous acidic methanol, is D-*erythro*-1,3-dihydroxy-2-amino-4-*trans*-octadecene.

There is still a question, however, as to whether the configuration of sphingosine *as it exists in the cerebrosides* is *erythro* or *threo*. Sphingosine has usually been obtained by acid hydrolysis of cerebrosides (8–12), a procedure which gives a mixture of bases from which pure sphingosine is obtained in very poor yield. From the crude mixture of bases obtained with anhydrous methanolic acids, Carter *et al.* (13) have isolated both the *erythro*- and *threo*-*O*-methyl ethers of sphingosine, and from aqueous methanolic hydrolysates of cerebrosides Seydel (14) obtained two different bases which subsequently were shown to be *threo*- and *erythro*-sphingosine. The isolation of both the *erythro* and the *threo* isomers of sphingosine from the hydrolysis of cerebrosides leaves open the question of the configuration of the sphingosine moiety of intact cerebrosides.

It has been shown that, in the case of simple *N*-acyl derivative of 1,2-

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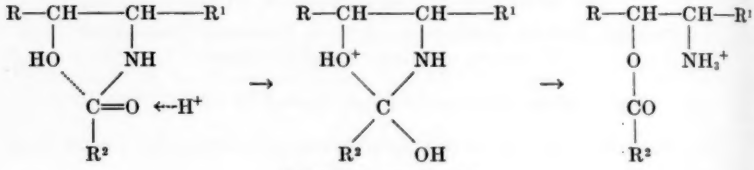
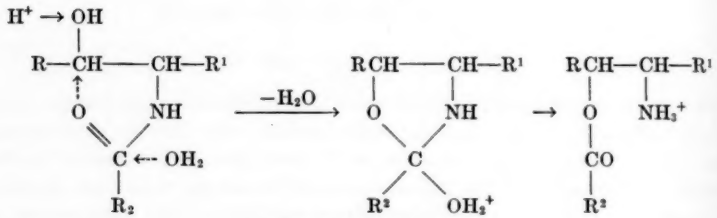
amino alcohols, acid hydrolysis results in an N → O shift of the acyl group and that the shift may occur either with retention or with inversion of configuration of the hydroxyl carbon atom. The accompanying mechanisms have been postulated to explain these reactions (15).



Retention mechanism



Inversion mechanism

It is particularly pertinent to the cerebroside problem that *erythro* acylamino alcohols usually give some inversion with the production of a mixture of two isomers, whereas the *threo* derivatives undergo hydrolysis with retention of configuration (15). These considerations point to an *erythro* configuration of the sphingosine moiety of cerebrosides, but experimental verification remains to be provided.

In order to approach this problem, it seemed desirable to apply a hydrolytic procedure which would eliminate the possibility of isomerization at carbon atom 3. To this end a pure cerebroside, phrenosine, was prepared from crude sphingolipide. Phrenosine is a representative cerebroside which is relatively easily separated in a pure form. A preliminary study was then made of the hydrolysis of dihydrophrenosine by anhydrous methanolic acids in the hope that destruction of the allylic system would minimize or eliminate inversion. The results were inconclusive. No *O*-methyl ethers of dihydrosphingosine were obtained, but the yield of *erythro*-dihydrosphingosine was only 50 per cent. Thus a considerable proportion of *threo*-sphingosine could have been produced, although it was not isolated.

Attention was next directed to the alkaline hydrolysis of phrenosine. By a modification of Klenk's procedure (16) psychosine (galactosidosphingosine) was obtained in good yields. Dihydropsychosine was readily obtained by catalytic reduction of psychosine. It seemed probable that acidic

hydrolysis of dihydropsychosine could be effected without inversion of the 3rd carbon, and this expectation was realized. Hydrolysis of psychosine with aqueous ethanolic hydrochloric acid gave a high yield of *erythro*-dihydrosphingosine, characterized as the tribenzoyl and triacetyl derivatives. No evidence could be obtained for the presence of *threo*-dihydrosphingosine in the hydrolysis product.

These data afford conclusive evidence that sphingosine as it exists in the phrenosine molecule has the *erythro* configuration. A study of other cerebrosides and of the sphingomyelins should be made to establish the generality of this relationship. However, it seems most likely that other sphingolipides will also have the *erythro* structure.

EXPERIMENTAL

Preparation of Phrenosine—Phrenosine (cerebron) was prepared from a crude sphingolipide mixture from spinal cord by a modification of the procedure of Klenk and Leupold (17). The sphingolipides were dissolved in 10 volumes of hot glacial acetic acid, and the solution was allowed to stand overnight at room temperature. The solution was filtered by suction, and the solid was washed with acetone and then dried in a desiccator connected to an aspirator. The dried material was fractionated once more in the same way. The treatment of 1 kilo of sphingolipides (P:1.33, 1.45 per cent) yielded 414 gm. of crude cerebrosides (P:0.035 per cent). Chromatography over alumina removed the remaining phosphorus-containing lipides. For this purpose, 1 part of crude cerebroside dissolved in 40 parts (by volume) of pyridine was percolated over 5 parts of alumina. The eluate was distilled under the reduced pressure to a small volume, and the cerebrosides were precipitated by adding acetone. In this way 220 gm. of purified cerebrosides, completely free from phosphorus, were obtained from 414 gm. of crude cerebroside. As the purified cerebroside mixture was slightly yellow and contained some kerasin, the recrystallization was repeated four times from 50 volumes of chloroform-methanol mixture (1:1) with charcoal. Phrenosine thus obtained (150 gm. yield) was a snow-white crystalline material which was essentially free of kerasin by the selenite plate test.

$C_{48}H_{93}NO_9$ (828.2). Calculated. C 69.05, H 11.24, N 1.69
Found. " 69.02, " 11.27, " 1.80
$[\alpha]_D^{23}$ +4.55° (0.2 gm. in 10 ml. of pyridine)

Preparation of Psychosine—Psychosine was prepared by a modification of Klenk's method (16). 8 gm. of phrenosine were dissolved in 80 ml. of warm dioxane, to which 80 ml. of 10 per cent barium hydroxide were added carefully so as not to cause excessive foaming. The reaction mixture was refluxed for 10 hours with occasional shaking to wash the adhering material down off the inside of the flask. The hot yellow-colored solution was

poured into 500 ml. of water, and the mixture was allowed to stand overnight. The precipitate was filtered by suction and washed with water. The solid was dried to constant weight (9.2 gm.). The dry solid was ground to a fine powder and was extracted with three 100 ml. portions of boiling ethanol. The ethanolic solutions were allowed to cool to room temperature and then filtered by suction. The 300 ml. of ethanolic filtrate thus obtained were concentrated under reduced pressure to give crude psychosine, which weighed 4.23 gm. (95 per cent yield from phrenosine). For purification, the crude psychosine was dissolved in about 80 ml. of ethanol and precipitated as the sulfate by dropwise addition of 1 N ethanolic sulfuric acid (freshly prepared) until the Congo red paper turned slightly blue. 80 ml. of ether were added to this solution to complete the precipitation of the sulfate, which was filtered and recrystallized from 1 liter of absolute ethanol. Psychosine sulfate was obtained as a beautiful white crystalline material (yield, 3.5 gm., 71 per cent of the theoretical; $[\alpha]_D^{20}$ −15.8° (0.7197 gm. in 10 ml. of pyridine)). The free base was prepared in the usual way from 2.8 gm. of psychosine sulfate by hydrolysis with 2.5 per cent barium hydroxide. The yield of psychosine was 2.55 gm. The product was a white powder, which sintered at 115°, turned yellow at 140°, and melted with decomposition at about 190°.

Hydrogenation of Psychosine—2.5 gm. of psychosine were hydrogenated in 300 ml. of 95 per cent ethanol at 80° under 500 lbs. per sq. in. of hydrogen with Raney's nickel as catalyst. 2.30 gm. of dihydropsychosine were obtained as a snow-white crystalline powder which gave no trans peak in the infra-red and which sintered at 135°, mostly melted at about 145°, and melted completely at 190° with decomposition. The same product was obtained in a micro hydrogenation apparatus at room temperature with the use of Adams' platinum catalyst.

$C_{24}H_{49}NO_7$ (463.64).	Calculated.	C 62.17, H 10.65, N 3.02
	Found.	" 61.79, " 10.35, " 2.83

Hydrolysis of Dihydropsychosine—2.0 gm. of dihydropsychosine were dissolved in 450 ml. of 1.6 N ethanolic hydrochloric acid, prepared by adding concentrated hydrochloric acid to ethanol. The solution was refluxed for 4 hours, during which the solution became a little yellowish. The reaction mixture was then concentrated under reduced pressure to dryness. The residue was dissolved in about 300 ml. of water and neutralized by adding 3 N sodium hydroxide solution to pH 9.5 and was then extracted with three 200 ml. portions of ether. The combined ether extracts were washed with water until the pH of the wash water was 7. The solution was dried for 2 hours over sodium sulfate and concentrated to dryness under reduced pressure, giving 1.30 gm. of crude dihydrosphingosine as a slightly yellowish powder (100 per cent yield).

Preparation of Tribenzoyldihydrosphingosine—Tribenzoyldihydrosphingosine was prepared by a modification of the procedure of Carter *et al.* (18). 0.3 gm. of crude dihydrosphingosine was dissolved in 5.0 ml. of freshly distilled pyridine and 1.2 ml. of freshly distilled benzoyl chloride were added dropwise. The reaction mixture was allowed to stand for 3 hours at room temperature, during which the solution became dark red and a white precipitate (pyridine hydrochloride) was formed. 40 ml. of ice-cold water containing 5 ml. of concentrated hydrochloric acid were added slowly. The white precipitate disappeared and a new white solid separated. This was filtered, washed thoroughly with water, and recrystallized twice from 30 ml. of absolute ethanol. Yield, 0.46 gm. of beautifully crystalline tribenzoyldihydrosphingosine (75 per cent) melting at 145–146°.

$C_{39}H_{51}NO_5$ (613.8).	Calculated.	C 76.31, H 8.38, N 2.28
	Found.	" 76.25, " 8.17, " 2.14

Preparation of Triacetyldihydrosphingosine—0.3 gm. of crude dihydrosphingosine was acetylated by the usual procedure (19), and gave 0.40 gm. of yellowish triacetyldihydrosphingosine (90 per cent yield) melting at 90–92°. Two recrystallizations from absolute ethanol gave 0.31 gm. of beautifully white crystalline triacetyldihydrosphingosine, melting at 100–102°. No *threo*-triacetylsphingosine could be isolated from the mother liquors.

$C_{24}H_{43}NO_8$.	Calculated.	C 67.41, H 10.61, N 3.28
	Found.	" 67.44, " 10.48, " 3.12

SUMMARY

An improved procedure has been developed for the preparation of psychosine from phrenosine. Dihydropsychosine (obtained by reduction of psychosine) on hydrolysis with ethanolic hydrochloric acid gives an excellent yield of *erythro*-dihydrosphingosine. No *threo* isomer could be detected in the hydrolysis product. These data establish that the sphingosine moiety of phrenosine has the *erythro* configuration.

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THE METABOLISM OF THE METHIONINE CARBON CHAIN IN THE INTACT RAT*

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In the present experiments the metabolism of the carbon chain of methionine has been studied in the intact rat by the administration of 2-, 3-, and 4-C^{14}-DL-methionine. The distribution of C^{14} in liver glycogen, serine, choline, and aspartic acid has been determined. The results are consistent with the metabolism of D- and L-methionine largely over the pathway of propionic acid catabolism and exclude the significant participation of certain other mechanisms.

EXPERIMENTAL

Methods and Materials

2-C^{14}-DL-Methionine was purchased from Tracerlab, Inc., and the California Foundation for Biochemical Research and purified by chromatography on a Dowex 50 column (2).

3- and 4-C^{14}-DL-methionine were synthesized from 1- and 2-C^{14}-acetate, respectively, by the following series of reactions

$$CH_3COONa \xrightarrow[(3)]{C_6H_5COCl} CH_3COCl \xrightarrow{Br_2} CH_2BrCOCl \xrightarrow{NaHCO_3} CH_2BrCOONa$$

$$\xrightarrow{CH_3SNa} CH_3SCH_2COOH \xrightarrow{LiAlH_4} CH_3SCH_2CH_2OH \xrightarrow[(4)]{SOCl_2} CH_3SCH_2CH_2Cl$$

$$NaC(NHCOCH_3)(COOEt)_2 \xrightarrow[(5)]{} CH_3SCH_2CH_2CH(NH_2)COOH$$

and purified by chromatography on Dowex 50 (2). The yield of methionine by this procedure was about 40 per cent. Paper chromatography of the amino acid with butanol-acetic acid solvent gave a single ninhydrin-

* This investigation has been supported by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council and by the Elisabeth Severance Prentiss Fund. A preliminary report of a portion of this work has appeared (1). The C^{14} used in this investigation was obtained on allocation from the United States Atomic Energy Commission.

positive and radioactive spot with an R_F identical with that of DL-methionine.

A detailed description of the synthetic procedure will be published elsewhere.

CH_3-C^{14}-DL-Methionine was prepared by the method of Melville *et al.* (6).

Determination of C^{14} Activity—The C^{14} activity of all the samples of Experiments 1 to 4 (Table I), with the exception of tetramethylammonium iodide, was determined by converting the carbon to $BaCO_3$ and counting with an end window Geiger-Müller counter. Tetramethylammonium iodide was plated and counted on stainless steel planchets, but for purposes of comparison the results have been expressed as the activity determined on barium carbonate. The activities of all the samples in Experiment 5 were determined by gas phase proportional counting (7).

Biological Experiments

C^{14}-labeled methionine was administered to rats subcutaneously together with oral doses of glycine or glucose. The latter substances were given to produce glycogen deposition and the glycine to stimulate serine synthesis as well. The general experimental data are summarized in Table I. Two animals from each group were kept in a metabolism cage for the collection of respiratory CO_2. At the end of the experiments the rats were anesthetized by intraperitoneal doses of 0.1 ml. of 10 per cent sodium Amytal solution per 100 gm. In each experiment the appropriate viscera were removed and pooled for the isolation of glycogen and other substances, as described below.

4-C^{14}-DL-Methionine and CH_3-C^{14}-DL-Methionine Experiments

Choline reineckate was isolated from the abdominal viscera as previously described (8) and degraded to trimethylamine (9, 10). The latter substance was converted to tetramethylammonium iodide for the determination of radioactivity (iodine, found, 63.5 per cent; calculated, 63.1 per cent).

After the extraction of phospholipides, glycogen was isolated from the liver by the procedure of Ostern and Hubl (11) and purified by precipitating it twice from 10 per cent trichloroacetic acid solution with alcohol. Glycogen was hydrolyzed and degraded by the procedure of Wood *et al.* (12) by utilizing *Lactobacillus casei*.

The neutral amino acid fraction of the liver hydrolysate, obtained as previously described (8), was chromatographed on Dowex 50 (2). Serine was separated from the fraction containing serine and threonine by precipitation as the *p*-hydroxyazobenzene-*p'*-sulfonate (13). After recrystallization from water, the product decomposed at 202–204° simultaneously with a sample of the authentic L-serine derivative and with a mixture of the two

samples. The distribution of the small amount of activity in serine was determined by degradation with periodate (8).

TABLE I

General Experimental Data on Rats

Experiment No.	Weight of fasted rats*	Methionine administered			Other substances administered‡	Length of experiment
		Type	Specific activity	Dose per 100 gm. and time of administration†		
	gm.		*c.p.m. per mg. labeled C*	*hrs.*		*hrs.*
1	150–170 (Average 161)	4-C^{14}	7.5×10^4	0.25 ml. of 0.3 M§ solution at 0, 3, 6, and 10 hrs.	Glycine, 2.0 ml. of 2.5 M solution per 100 gm.	14
2	155–170 (Average 163)	CH_3-C^{14}	7.5×10^4	0.25 ml. of 0.3 M§ solution at 0, 3, 6, and 10 hrs.	Glycine, 2.0 ml. of 2.5 M solution per 100 gm.	14
3	174–190 (Average 183)	3-C^{14}	1.75×10^5	0.18 ml. of 0.3 M§ solution at 0 hrs.	Glucose, 2.5 ml. of 1.35 M solution per 100 gm.	4
4	150–158 (Average 154)	2-C^{14}	1.43×10^5	0.18 ml. of 0.3 M§ solution at 0 hrs.	Glucose, 2.5 ml. of 1.35 M solution per 100 gm.	4
5	149–151 (Average 150)	2-C^{14}	1.09×10^7	0.30 ml. of 0.15 M‖ solution at 0 hrs.	Glucose, 2.5 ml. of 1.35 M solution per 100 gm.	4

* Six rats were used in Experiments 1, 2, and 5 and four in Experiments 3 and 4. The rats used in Experiments 1 and 2 were fasted for 48 hours, the others for 24 hours.

† Subcutaneous.

‡ By stomach tube.

§ Warmed before administration, supersaturated at room temperature.

‖ In 0.08 M NaCl.

37 per cent of the C^{14} of 4-C^{14}-methionine and 28 per cent of that of the Me-C^{14} compound were recovered in the respiratory CO_2. The labeling of the serine, choline, and glycogen is shown in Tables II and III. Very

little of the 4-C^{14}-methionine activity was incorporated into the serine-β-carbon or choline methyl groups. Glycogen was labeled in all carbons, with equal activity in the 1,6 and 2,5 positions. CH_3-C^{14}-Methionine produced high labeling of the serine-β-carbon and of all of the glycogen carbon atoms

TABLE II

Distribution of Isotope in Liver Glycogen after Administration of C^{14}-Labeled Methionine

Experiment No.	Type of C^{14}-methionine administered	Average specific activity of glucose carbons*	Labeling of the carbon fractions of glucose*			
			1†	1,6†	2,5†	3,4†
1	4-C^{14}			1480	1500	316
2	CH_3-C^{14}			970	740	228
3	3-C^{14}			270	307	71
4	2-C^{14}			2	2	232
5	2-C^{14}	2900‡ 2820§	0.2			

* C.p.m. per mg. of carbon.
† Positions of the carbon atoms in glucose.
‡ Determined on glucose phenylosazone.
§ Determined on glucose *p*-nitrophenylhydrazone.

TABLE III

*Labeling of Serine, Choline, and Aspartic Acid by C^{14}-Methionine**

Type of methionine administered	Serine			Choline	Aspartic acid		
	β-C	α-C	COOH-C	CH_3-C	Average	2,3	1,4
4-C^{14}	6	6	6	0			
CH_3-C^{14}	1700	1	2				
2-C^{14}					819	28	1610†

* In c.p.m. per mg. of carbon.
† Calculated.

with significantly higher activity in the 1,6 than in the 2,5 position, as shown in Table II.

3-C^{14}-DL-Methionine and 2-C^{14}-DL-Methionine Experiments

In the 3-C^{14}-methionine and first 2-C^{14}-methionine experiments (Experiments 3 and 4), liver glycogen was isolated by the method of Good, Kramer, and Somogyi (14) and purified and degraded as described in the preceding section. The results are presented in Table II. 2-C^{14}-Methionine labeled the glycogen in the 3,4 carbon, whereas 3-C^{14}-methionine produced gly-

cogen labeled in all positions with approximately equal activity in the 1,6 and 2,5 carbons.

In the second 2-C^{14}-methionine experiment (Experiment 5) liver glycogen was isolated, and the liver protein was hydrolyzed as in Experiments 1 and 2. Portions of the glucose were converted to the phenylosazone and *p*-nitrophenylhydrazone derivatives which were recrystallized (m.p. 205° and 190°, respectively) and oxidized to CO_2 for the determination of the average specific activity of the glucose carbon. A third portion of the glucose was partially degraded by fermentation with *Leuconostoc mesenteroides*, which converts the 1 carbon to CO_2 (15). The dicarboxylic amino acids were separated from the hydrolysate by the procedure of Cannan (16). After removal of the glutamic acid as the hydrochloride, the aspartic acid was precipitated as the copper salt which was recrystallized and freed of copper by the usual procedure. Aspartic acid was purified by chromatography on a 70 cm. Dowex 50 column (2). Although the amino acid was pure, as indicated by a symmetrical elution curve of radioactivity and amino acid concentration (17), the sample was subjected to chromatography on a 40 cm. Amberlite IR-4B (acetate) column (18). The Amberlite introduced a colored impurity into the aspartic acid which was removed by rechromatographing on Dowex 50. Part of the aspartic acid was oxidized to CO_2 for determination of the average specific activity of the carbon. The remainder was degraded with hypochlorite, which converted carbon atoms 2 and 3 to acetaldehyde (19). The acetaldehyde-2,4-dinitrophenylhydrazone was oxidized to CO_2 to determine the average C^{14} activity of the acetaldehyde carbon.

15 per cent of the C^{14} of 3-C^{14}-methionine was recovered in the respiratory CO_2, and 32 and 42 per cent of the C^{14} were recovered after the administration of the 2-C^{14} compound in Experiments 4 and 5, respectively. The C^{14} activity of the liver glycogen and aspartic acid and the distribution of this activity are shown in Tables II and III. The glycogen and aspartic acid possessed considerable C^{14} activity, but very little was located in the 2 and 3 carbons of the aspartic acid or the 1 carbon of the glucose.

The conversion of D- and L-methionine to propionate or a propionic acid derivative, as shown in Scheme 1, is proposed as the principal pathway of methionine metabolism in animals. Previous studies have demonstrated the occurrence of the various steps of this scheme.

L-Methionine → α-ketobutyrate → propionate

⇅

α-Keto-γ-methiolbutyric acid

↑

D-Methionine

SCHEME 1. Proposed principal mechanism of methionine metabolism

The conversion of D- to L-methionine is indicated by the ability of D-methionine to support the growth of rats receiving a diet deficient in methionine and cystine (20) and by the existence of enzymes capable of deaminating D-methionine (21) and reaminating the corresponding keto acid to L-methionine (22–26).

α-Ketobutyric acid is formed from methionine by a number of mechanisms. Carroll *et al.* (27) showed that α-ketobutyrate is a product of L-cystathione cleavage by rat liver, and Binkley (28) observed that a purified cystathionase preparation degraded DL-homocysteine and DL-methionine with the formation of ammonia and an equivalent amount of H_2S and methyl mercaptan, respectively. α-Ketobutyrate was identified as a product of the desulfurization of homocysteine. Evidence for the formation of α-aminobutyric acid[1] (29–31) and homoserine[1] (31) from DL-methionine and for the conversion of D- (21) and L-α-aminobutyric acid (22, 23, 26) and L-homoserine (27, 32) to α-ketobutyrate has also been reported.

The conversion of α-ketobutyric acid to propionate is supported by reports that it is rapidly and oxidatively decarboxylated by pigeon brain (33, 34), and that DL-α-aminobutyrate is converted to α-ketobutyric and propionic acids by rat liver homogenate (35).

The results of the present experiment are in full accord with the postulate that carbon atoms 2, 3, and 4 of D- and L-methionine are metabolized in animals largely via propionic acid. The types of glycogen produced by the C^{14}-DL-methionines (Table II) were identical with those which would have been produced on conversion of the 2, 3, and 4 carbons to propionic acid. The 2-C^{14}-DL-methionine, like 1-C^{13}-propionate (36), introduced labeling only into the 3,4 position of the glycogen and 3-C^{14}- and 4-C^{14}-DL-methionine, like 2-C^{13}- and 3-C^{13}-propionate (36), introduced labeling into all the carbons of the glucose with roughly equal labeling in carbon atoms 1,6 and 2,5.

It is recognized that the present data do not provide conclusive proof of the principal metabolism of methionine by the proposed scheme (Scheme 1). However, the occurrence in the rat of two other mechanisms is eliminated. One is the direct conversion of methionine to aspartic acid, a pathway suggested by the formation of homoserine from DL-methionine in rats (31) and the existence of a mechanism for the transformation of L-homoserine to L-aspartic acid in yeast (38–40). By this process, 2-C^{14}-methionine would label the 2 carbon of aspartic acid and the 1,6 and 2,5 positions of the liver glycogen. While the aspartic acid and glycogen were heavily labeled, negligible C^{14} was located in the 2 and 3 carbons of aspartic acid or in the 1 position of the glucose (Tables II and III). These results contrast with the report of Marshall and Friedberg (41) that the activity of 2-C^{14}-

[1] The optical identity of the amino acid was not reported.

methionine is primarily incorporated into the non-carboxyl carbons of aspartic acid in mice.

The metabolism of methionine via conversion of the carbon chain to pyruvate and formaldehyde (from the γ-carbon) has also been excluded by the present data.[2] According to this pathway, 4-C^{14}-methionine would be expected to form C^{14}-formaldehyde and to label the serine-β-carbon and choline methyl groups (42–44). Little or no C^{14} was found in these moieties; the administration of an identical dose of methyl-C^{14}-methionine labeled the serine-β-carbon very heavily (Table III). In addition, this mechanism would produce 2-C^{14}-pyruvate from 2-C^{14}-methionine and would be expected therefore to introduce C^{14} into the 1,6 and 2,5 carbon atoms of the liver glycogen as is observed with 2-C^{14}-lactate (45). The results of *Lactobacillus casei* degradation of the glycogen indicated that there was no such labeling. A more exact determination of the C^{14} in the 1 carbon was made in Experiment 5 by gas phase proportional counting of the CO_2 from this position. The specific activity of the 1 carbon of the glucose was very low (Table II).

The indication for the formation of propionic acid as a major intermediate of methionine catabolism contributes to the evidence that propionate is an amino acid metabolite of considerable quantitative importance. Coon *et al.* (46) have presented data indicating that propionic acid is formed from the 2,3- and 3′-methyl carbons of isoleucine in rats, and Fones *et al.* (47), Peterson *et al.* (48), and Kinnory *et al.* (49) have obtained evidence for the formation of propionate from the isopropyl group of L-valine in this animal. Lien and Greenberg (50) have also shown that α-ketobutyrate is formed from DL-threonine in rat liver homogenate. However, it appears from the studies of Meltzer and Sprinson (51) that this pathway is not of major quantitative significance in the metabolism of threonine in the normal rat.

The assistance of Dr. John R. Murphy with the gas phase proportional counting and of Dr. Murphy and Dr. J. A. Muntz in the *L. mesenteroides* degradation of glucose is gratefully acknowledged.

SUMMARY

2-C^{14}-, 3-C^{14}-, and 4-C^{14}-methionine have been administered to fasting rats, and the isotope distribution in the liver glycogen has been determined. 2-C^{14}-Methionine labels the glycogen solely in the 3,4 carbon, whereas 3-C^{14}- and 4-C^{14}-methionine label the glycogen in all positions with equal labeling in the 1,6 and 2,5 carbons. It has also been found that 4-C^{14}-methionine does not label the serine-β-carbon and choline methyl groups

[2] This conversion was conceived to occur via α-keto-γ-hydroxybutyric acid which is formed from pyruvate and formaldehyde in liver (37).

and that 2-C^{14}-methionine labels aspartic acid principally in the carboxyl carbon. The implications of these findings are discussed.

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THE QUANTITATIVE MEASUREMENT OF γ-GLUTAMYL TRANSPEPTIDASE ACTIVITY*

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It was observed by Hanes *et al.* (11, 12), using paper chromatographic procedures, that kidney extracts were able to catalyze transpeptidation reactions involving γ-glutamyl peptides. Independently, Kinoshita and Ball (16, 17) observed the same reaction by a method which permitted quantitative measurement of the product formed. The method of the latter workers was limited in its usefulness, however, in that, though any γ-glutamyl peptide could be employed as the donor, only arginine could be used as the acceptor of the glutamyl moiety. We have, therefore, sought means for the quantitative measurement of this reaction which would permit a study of amino acids other than arginine. We describe here two procedures. They both are dependent, however, upon the use of glutathione as the γ-glutamyl donor.

One method is manometric and this permits the reaction to be readily followed throughout its time-course. It is based upon the release of cysteinylglycine which occurs in the reactions catalyzed by the enzyme. During the initial stages of the reaction these may be represented as shown by the following equations:

$$\text{Glutathione} + H_2O \rightarrow \text{glutamic acid} + \text{cysteinylglycine} \quad (1)$$

$$\text{Glutathione} \rightarrow \text{pyrrolidonecarboxylic acid} + \text{cysteinylglycine} \quad (2)$$

$$\text{Glutathione} + \text{glutathione} \rightarrow \gamma\text{-glutamylglutathione} + \text{cysteinylglycine} \quad (3)$$

$$\text{Glutathione} + \text{amino acid} \rightarrow \gamma\text{-glutamyl peptide} + \text{cysteinylglycine} \quad (4)$$

The amount of cysteinylglycine formed is determined by measuring the CO uptake which occurs in the following reaction:

$$2\ \text{Cysteinylglycine} + Fe^{++} + 2CO \rightarrow (\text{cysteinylglycine})_2Fe(CO)_2 \quad (5)$$

The method has its origin in the observations of Cremer (8) and Kubowitz (18) that, whereas cysteine readily forms an iron-carbon monoxide chelate, glutathione is relatively inactive in this respect. As will be shown here,

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cysteinylglycine resembles cysteine in its ability to react readily with iron and carbon monoxide.

The second method employed to follow quantitatively γ-glutamyl transpeptidase activity is based upon the measurement of the disappearance of glutathione and employs the alloxan procedure of Patterson and Lazarow (22)[1] to measure glutathione. In this procedure, use is made of the ability of alloxan to react with glutathione to form a compound with a maximal absorption at 305 mμ.

Methods and Materials

Manometric Method—The reaction is followed by the conventional Warburg manometric procedure at a temperature near 38°. Vessels with two side arms are usually employed. In the experiments to be described here, the main chamber has contained 2.0 ml. of 0.1 M borate buffer, pH 9.0, the amino acid or other γ-glutamyl acceptor, and water to make the total fluid volume 3.0 ml. after the contents of both side arms have been dumped. In one side arm is placed the enzyme preparation, and in the other 0.2 ml. of a 0.1 M solution of glutathione (20 μmoles) and 0.15 ml. of a freshly prepared solution of 0.05 M $FeSO_4$ (7.5 μmoles). We have preferred to employ the isoelectric form of glutathione and of amino acids or other γ-glutamyl acceptors rather than to titrate them to the pH of the medium. The addition of these components and the ferrous sulfate to 0.1 M borate buffer of pH 9.0 in the amounts and under the conditions described herein causes the pH to shift to a value lying in the range 8.6 to 8.7, the optimum for the reaction. The vessels are usually filled in the order given, and are gassed with CO from a commercial cylinder. It may sometimes be necessary to pass the gas through pyrogallol to remove O_2. The glutathione and iron may be dumped as soon as the vessels are gassed. They must not be added to the alkaline buffer in the presence of oxygen since rapid oxidation of both the ferrous salt and the SH group of glutathione occurs under alkaline conditions. The vessels are placed in a constant temperature bath and allowed to come to equilibrium. There may be a slow uptake of CO for the first 10 to 20 minutes due to the formation of the ferrous chelate of glutathione. This usually amounts to not more than 20 μl. of CO. The enzyme solution is then tipped in to start the reaction. Reaction vessels with a single side arm may be employed. Then the enzyme is placed in the main chamber and the reaction started by dumping the mixture of glutathione and $FeSO_4$.

This method is limited to use in the alkaline pH range, due to the fact that the chelation of cysteinylglycine with iron and CO becomes a limiting

[1] We are indebted to Dr. Lazarow for making the details of this method available to us before publication.

factor as the pH approaches neutrality. The method appears to work successfully as low as pH 8.3 in borate buffer, though we have mainly employed the method at a pH nearer to 9.0. The limiting amount of ferrous sulfate which can be successfully kept in solution under the conditions of the experiment appears to be about 7.5 μmoles per 3 ml. of borate buffer. This amount of iron theoretically permits the chelation of 15 μmoles of cysteinylglycine and the uptake of 336 μl. of CO. It is obviously necessary, therefore, to keep this point in mind in the design of the experiments.

Alloxan Method—This reaction was run in Thunberg tubes in an atmosphere of N_2 purified by passage over heated copper filings. Each tube contained 2.0 ml. of 0.1 M buffer, enzyme solution, and water or amino acid solution to make a volume of 2.7 ml. In the side arm was placed 0.3 ml. of a glutathione solution containing usually 20 μmoles. The contents of the tubes were brought to 38° and the glutathione dumped to start the reaction. At the desired time interval tubes were opened and 0.5 ml. of the contents immediately pipetted into a mixture of 3.5 ml. of H_2O and 1.0 ml. of 25 per cent metaphosphoric acid. The precipitated protein was filtered and the glutathione content of the filtrate determined by the method of Patterson and Lazarow. To 0.25 ml. of filtrate, the following reagents were added, with mixing and in the order given: 0.25 ml. of 5 per cent metaphosphoric acid, 1.2 ml. of H_2O, 1.0 ml. of 0.1 M alloxan (freshly prepared), 1.0 ml. of 0.5 M phosphate buffer, pH 7.5, and 0.3 ml. of 1.0 M NaOH, the last two reagents being added to each tube in rapid succession. The final pH of the mixture should be close to 7.5. Each tube is allowed to stand exactly 6 minutes after the above reagents are added and then 1.0 ml. of 1.0 M NaOH is added with mixing. The extinction at 305 mμ is then determined in a Beckman model DU spectrophotometer. A glutathione standard and appropriate blanks are also run concurrently. In agreement with Patterson and Lazarow (21), we have obtained density readings at 305 mμ lying between 0.46 and 0.50 when a 1 cm. cell and a glutathione concentration of 1 mg. per 100 ml. of final reaction mixture are used. We have also confirmed their finding that cysteinylglycine undergoes no appreciable reaction with alloxan to form a product absorbing in this region. The average absorption at 305 mμ that we have obtained in three experiments with cysteinylglycine was less than 3 per cent of that given by an equivalent amount of glutathione.

pH Determination—All values for pH were determined by means of a glass electrode at room temperature. The instrument was calibrated against standard buffers.

Materials—The glutathione, cysteine, alanine, and glycylglycine employed were commercial samples, and the ferrous sulfate was a commercial sample recrystallized so as to yield the monohydrate. The cysteinylglycine

was a white crystalline preparation synthesized in the laboratories of Dr. John Sheehan, to whom we are indebted for the sample used. When purified enzyme was used, it was prepared from beef kidney cortex by the method of Kinoshita and Ball (17) with slight modifications. Kidney homogenates were prepared by blending fresh kidney with buffer solution or isotonic KCl in a Potter-Elvehjem type (23) homogenizer. They were usually employed at a final dilution of 1 gm. of tissue per 100 ml.

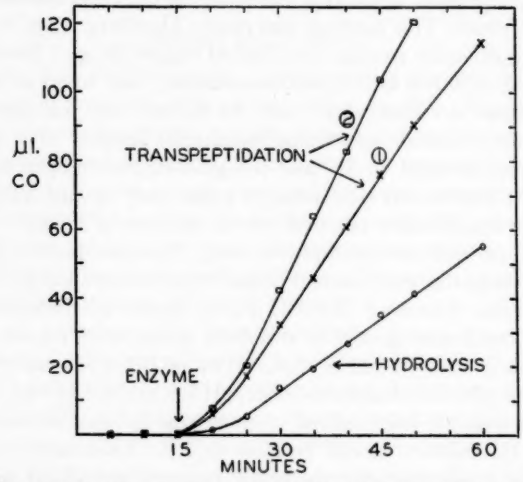

FIG. 1. Manometric measurement of γ-glutamyl transpeptidase activity. Each flask contained 1.25 mg. of purified enzyme, 7.5 µmoles of $FeSO_4$, 20 µmoles of glutathione, 2.0 ml. of 0.1 M borate buffer, pH 9.0, in a total volume of 3.0 ml. L-Alanine was present in those flasks in which transpeptidation was measured; Curve 1, 10 µmoles; Curve 2, 20 µmoles; temperature, 37.8°. The pH of the contents of all the vessels after mixing fell within the range of 8.6 to 8.7.

Results

The type of data obtained with the manometric method is shown by the results presented in Fig. 1. In this experiment a purified kidney enzyme preparation was tipped into the flask to start the reaction. One flask contained only glutathione with ferrous iron and buffer. In this flask cysteinylglycine release may be brought about by the reactions shown in Equations 1 to 3. Since the method does not permit us to distinguish between the amount of breakdown resulting from each of these possible pathways, we have simply labeled this reaction in Fig. 1 as "Hydrolysis." In the other two flasks, L-alanine was also present in total amounts of 10 and 20 µmoles per vessel, respectively. Here cysteinylglycine production can theoreti-

cally occur by any one of the four reactions shown in Equations 1 to 4. Since again we are not able to determine the amount of cysteinylglycine formed in each of the four possible pathways, we have chosen to label these curves in Fig. 1 "Transpeptidation." It will be noted that in all the flasks there is a slight lag period in CO absorption after dumping the enzyme. This lag period is usually of about 5 minutes duration. The reaction then proceeds linearly for a period of about 30 minutes. For the purpose of making comparative rate measurements, we prefer to use the rate during this linear period of the reaction. For example, by this means, the data presented in Fig. 1 give a CO uptake rate for hydrolysis of 86 μl. per hour, while that for transpeptidation is 175 and 250 μl. per hour with 10 and 20 μmoles of L-alanine, respectively. Though not shown in Fig. 1, the rate with 20 μmoles of DL-alanine per vessel has been found to be the same as that with 10 μmoles of L-alanine. This indicates that the D form of the amino acid neither participates in the reaction nor inhibits it.

Several questions must now be answered as to the validity of this manometric procedure for following the rates of the enzymatic reactions under study. We will now endeavor to deal with these in turn. First, the question may be raised as to whether cysteinylglycine is capable of reacting with CO and ferrous iron or whether the reaction observed depends upon the subsequent release of cysteine from the dipeptide by further enzymatic action. It can be shown that cysteinylglycine will chelate more readily than cysteine with ferrous iron and carbon monoxide. We have compared the rate of uptake of CO by cysteine and cysteinylglycine after dumping them into a borate buffer at pH 9.0 containing ferrous iron at a temperature of 37.8°. By using concentrations of these two components in the range 4 to 7 μmoles per vessel, it was found that 50 per cent of the final equilibrium value was reached in the case of cysteinylglycine in about 3 minutes. In the case of cysteine the reaction was about one-fifth as fast. The final equilibrium value for the reaction of CO with cysteinylglycine under the conditions of our experiments reaches approximately 85 per cent of theoretical. The relation between total CO absorbed and the amount of cysteinylglycine added to the flask is shown in Fig. 2. If 1 mole of CO were absorbed for each mole of cysteinylglycine formed, then the relation shown by the dashed line in Fig. 2 would be expected. The amount of cysteinylglycine added was determined by iodometric assay of its reducing equivalents. Under similar conditions we have found cysteine to reach 90 per cent of theoretical equilibrium values, which confirms the findings of Cremer (8).

An answer to whether the ferrous salt influences the reaction has been approached in the manner shown by the experiment in Fig. 3. In this experiment the enzymatic reaction has been started but the ferrous iron

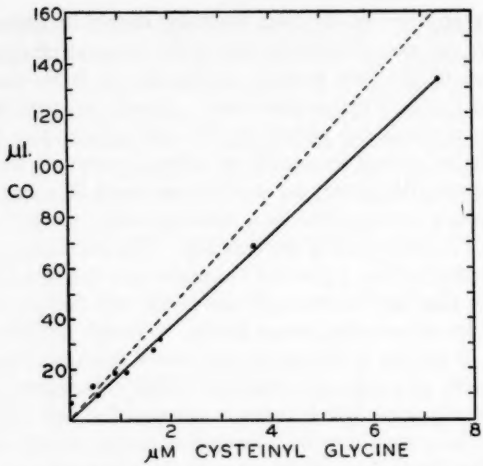

FIG. 2. Total CO uptake by cysteinylglycine. Each flask contained 7.5 μmoles of $FeSO_4$, 2.0 ml. of 0.1 M borate buffer, pH 9.0, and the amount of cysteinylglycine indicated, in a total volume of 3.0 ml.; temperature, 37.8°. The dashed line indicates a theoretical uptake.

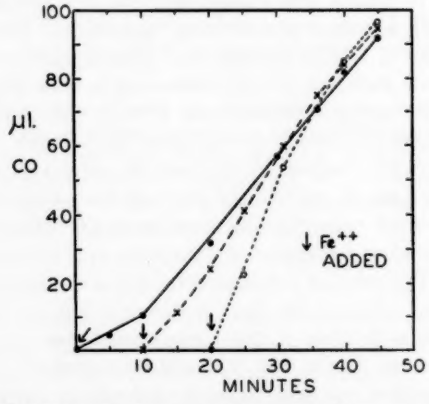

FIG. 3. Enzyme activity in presence and absence of ferrous sulfate. Each flask contained 1.87 mg. of purified enzyme, 20 μmoles of glutathione, 20 μmoles of L-alanine, 7.5 μmoles of $FeSO_4$, and 2.0 ml. of 0.1 M borate buffer, pH 9.0, in a total volume of 3.0 ml. The enzyme was tipped into the main chamber at zero time in all the flasks; the $FeSO_4$ was added at the times indicated on the graph by the arrows. The pH of the mixture without added iron was 8.71 and fell to 8.63 with the addition of the ferrous sulfate.

has been added at different time intervals during the course of the reaction. It can be seen that the addition of the iron salt either 10 or 20 minutes after the initiation of the enzyme reaction leads to no change in the total amount of CO eventually absorbed, and that by the end of the 40 minute period the reaction is proceeding in all flasks at the same rate. This indicates that

TABLE I

Comparison of Data Obtained by Manometric and Alloxan Methods

Experiment No.	Glutathione	L-Alanine	Time	Method	
				Cysteinylglycine formed	Glutathione used
	μmoles	*μmoles*	*min.*	*μmoles*	*μmoles*
1	20	0	30	3.1	3.1
	40	0	30	3.7	4.2
2	20	20	15	3.4	3.3
			30	6.9	6.7
			30		6.3 (CO)*
3	20	100	7.5	1.5	1.5
			15	3.4	4.0
			30	6.9	6.2
4	20	0	15	1.5	1.5
			30	3.0	3.4
			45	4.4	4.5
			60		6.2
5	20	100	15	2.3	2.8
			30	4.4	4.8
			45	6.5	6.4

* The reaction was carried out in an atmosphere of CO instead of N_2. Experiments 1 to 3 were performed with purified enzyme, while in Experiments 4 and 5 rat whole kidney homogenate was employed. Temperature: manometric method, 37.8°; alloxan method, 38.0°. In all cases, 2.0 ml. of 0.1 M borate buffer, pH 9.0, were used per 3.0 ml. of reaction mixture. The initial pH of the final mixture in all cases as measured with the glass electrode fell within the range 8.6 to 8.7. The amount of enzyme preparation employed varied in the different experiments, so that a comparison of the results is not valid.

the ferrous iron is without any significant effect upon the kinetics of the enzyme reaction.

Some answers to the questions already raised and to certain others have been provided by a comparison of the results obtained by the manometric method with those furnished by the alloxan procedure. In Table I are data obtained by both methods under comparable experimental conditions. For presentation in Table I, we have converted the manometric data into micromoles of cysteinylglycine formed by dividing the microliters of CO

absorbed in a given time interval by 19. The value 19 represents 85 per cent of the theoretical value of 22.4 μl., and its use is based upon the data presented in Fig. 2. An inspection of Table I will show that the values obtained by the two methods under a variety of experimental conditions are for the most part in fair agreement. There is a tendency for the alloxan method in some instances to give values 10 per cent or so higher than those obtained by the manometric procedure, and also to yield values not as linear with time. In this connection it should be noted that high results

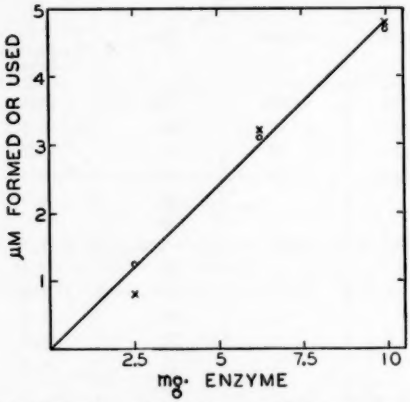

FIG. 4. Rate of hydrolysis of glutathione at different enzyme concentrations. The results are expressed in micromoles of glutathione destroyed by the alloxan method (×) at 38°, or in micromoles of cysteinylglycine formed in the manometric procedure (○) at 37.8°, during a 40 minute incubation period in the presence of the stated amounts of a purified enzyme preparation. In all cases, 20 μmoles of glutathione were used and no γ-glutamyl acceptor was added; 2.0 ml. of 0.1 M borate buffer, pH 9.0, in a total volume of 3.0 ml.; initial pH of mixtures, 8.64 to 8.72. All other experimental procedures were as described in the text.

will be obtained in the alloxan method if any oxidation of glutathione occurs during the experimental procedure. The alloxan method measures only the reduced form of glutathione, and hence any glutathione which is oxidized will contribute to the amount which is measured as having disappeared. Autoxidation of glutathione can be a particularly dangerous source of error at pH values near 9.0, as used in these experiments. Also, since the alloxan method depends upon a measurement of the difference between the initial concentration of glutathione and that remaining at a given time interval, it is least reliable when used to measure the disappearance of small amounts of glutathione.

The data in Table I also indicate that there is no essential difference in the results obtained at this pH by the two methods, whether one employs

a purified enzyme preparation or a crude kidney homogenate. This suggests that kidney peptidases other than γ-glutamyl transpeptidase play at best a negligible quantitative rôle in the results obtained. Also, as seen in Table I, the substitution of CO for N_2 has little effect upon the reaction as measured by the alloxan method. A similar lack of effect of CO upon the enzymatic reaction has been demonstrated by the use of the method of Kinoshita and Ball (17).

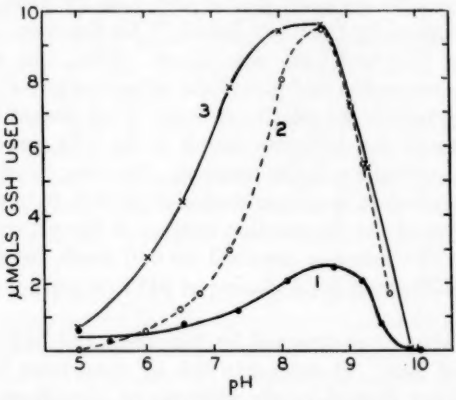

FIG. 5. pH-activity curve for hydrolysis and transpeptidation. Curve 1, 20 μmoles of glutathione per vessel; Curve 2, 20 μmoles of glutathione + 100 μmoles of L-alanine per vessel; Curve 3, 20 μmoles of glutathione + 20 μmoles of glycylglycine per vessel. All the values were determined by the alloxan method at 38° with an incubation period of 30 minutes. Each 3 ml. of reaction mixture contained, in addition to the components listed, 2 ml. of 0.1 M buffer and 0.4 ml. of 1:100 homogenate of rat kidney. Acetate buffer was used in the region of pH 5.0, phosphate buffer in the range, pH 5.5 to 7.4, tris(hydroxymethyl)aminomethane buffer in the region of 8.0, and borate buffer at the remaining pH values. All pH values recorded in Fig. 5 were determined by means of the glass electrode at the end of the incubation period.

The data presented in Fig. 4 show that the rate of the reaction is proportional to the enzyme concentration and that the two methods again give concordant results. Here the manometric data have been corrected as described for the data presented in Table I.

pH-Activity Curve—By using the alloxan method, the activity of the enzyme has been measured at different pH values and when the reaction mixture contained (1) glutathione alone, (2) glutathione plus glycylglycine, and (3) glutathione plus L-alanine. In these experiments, 20 μmoles of glutathione and glycylglycine and 100 μmoles of L-alanine were employed in a total volume of 3.0 ml. The conditions of the experiments in all three cases were the same, so that the results in Fig. 5 are directly comparable

with one another. For example, at the optimal pH, the rate of the reaction is the same for glycylglycine and L-alanine if the latter is present at a concentration 5 times that of the former and this rate is 3.8 times that for hydrolysis. It will be noted that, though the optimal pH for the reaction under all conditions lies within the region of pH 8.5 to 8.8, there is a spread of the curves on the acid side of this optimal region. For example, the pH value at which 50 per cent of the optimal activity is reached on this acid side is 6.65 for glycylglycine and 7.60 for L-alanine. The pK values at 25° as given by Cohn and Edsall (7) for the amino groups of these compounds are 8.15 and 9.69, respectively. Thus, the position of the pH-activity curves on the acid side of the pH optimum for these two compounds directly reflects the relative strength of the ionization constants of their amino groups and indicates that it is the NH_2 form of these compounds which participates in the reaction. By using the pK values given above, one can calculate the concentration of the NH_2 form of glycylglycine and L-alanine present in the reaction mixture at the pH values for 50 per cent activity. The values so obtained are 0.67 μmole for glycylglycine at pH 6.65, and 0.72 μmole for L-alanine at pH 7.60 per 3.0 ml. of reaction mixture.

The pH-activity curve reported by Kinoshita and Ball (17) resembles those presented here. It was obtained by measuring the amount of γ-glutamylarginine formed in the presence of glutathione and arginine under experimental conditions similar to those employed here. On the acid side of the optimal pH, their curve lies intermediate to those given here for glycylglycine and L-alanine. The pK value at 25° for the arginine amino group is reported to be 9.04 (7), which is thus also intermediate to those given for glycylglycine and alanine. From the data of Kinoshita and Ball it can be calculated that 50 per cent of the optimal activity is reached on the acid side at pH 7.10.

Fruton and his coworkers (10, 15), in their studies on reactions catalyzed by a variety of proteolytic enzymes, were the first to point out the pH dependence of transamidation and its relation to the pK of the replacement agent. The results presented in Fig. 5 serve to reemphasize the need for a careful choice of pH and concentrations in making a study of the relative reactivities of a group of compounds as replacement agents in transpeptidation reactions. As can readily be seen from Fig. 5, any quantitative conclusions drawn as to the relative merits of L-alanine *versus* glycylglycine as γ-glutamyl acceptors will vary greatly, depending on the pH value chosen for making such a comparison.

The pH-activity curve obtained for the disappearance of glutathione in the absence of an added γ-glutamyl acceptor (Fig. 5) was obtained with a rat kidney homogenate. Similar results were obtained with a purified

beef kidney enzyme preparation or sheep kidney homogenates. The shape of the "hydrolysis" curve, though it resembles those obtained in the presence of L-alanine or glycylglycine, seems to taper off more slowly in the acid region. This may be a reflection of the interplay of the three types of reactions which are postulated to occur (see Equations 1 to 3) and which may be masked or suppressed in the presence of an added acceptor. Two of these reactions (Equations 2 and 3) postulate the participation in the reaction of the amino group of the glutamyl moiety of glutathione. In glutathione the pK of this amino group has been assigned a value of 8.66 and the SH group a value of 9.12 (7), though Calvin (6) has recently raised the question as to whether these values should be interchanged (see also Benesch and Benesch (3)). If, as suggested by the results with L-alanine and glycylglycine, it is the uncharged amino group which participates, then here also it would seem that the increase in the rate of disappearance of glutathione which occurs as the pH increases might be attributed largely to the increasing participation of the reactions in Equations 2 and 3.

We should clearly state that we have no evidence other than the formation of cysteinylglycine or the disappearance of glutathione to present at this time for the occurrence of any of the reactions given in Equations 1 to 3. Evidence for the formation of pyrrolidonecarboxylic acid, as shown in Equation 2, was first presented by Woodward and Reinhart (26). These workers found that both pyrrolidonecarboxylic acid and glutamic acid were formed during the incubation of glutathione with rat kidney extracts. The relative amounts of these two compounds formed varied with the pH, glutamic acid predominating acid to pH 6.6, and pyrrolidonecarboxylic acid predominating on the alkaline side of this pH. Recently Fodor *et al.* (9) have also reported that pyrrolidonecarboxylic acid is a product of glutathione breakdown. On the other hand, Hird and Springell (14) reported that they have not been able to detect pyrrolidonecarboxylic acid when glutathione is incubated with sheep kidney preparations. These workers state that "glutathione is completely hydrolysed to glutamic acid at pH 6.0, 7.4, and 8.5; the completion of the reaction taking longer, however, at the higher pH values." The optimal pH for the conversion of glutathione to glutamic acid is reported by them to be 6.0. If pyrrolidonecarboxylic acid is not formed and glutamic acid production decreases with increasing pH, then the increase in rate of the reaction that we have observed as the pH increases would have to be attributed to Equation 3. In this reaction γ-glutamylglutathione would be formed initially. We have no knowledge as to whether this compound reacts with alloxan in the same manner as glutathione. However, the fact that the alloxan method and the manometric method (Table I) yield similar values for the so called

hydrolysis reaction at pH 8.6 to 8.7 suggests that, if γ-glutamylglutathione is present, it is measured as glutathione.

It should perhaps be mentioned that, in addition to the reactions already considered, there is the possibility that some disappearance of glutathione as measured by the alloxan method could be brought about by the following reaction:

$$\text{Glutathione} + H_2O \rightarrow \gamma\text{-glutamylcysteine} + \text{glycine}$$

In the alloxan procedure, γ-glutamylcysteine is reported (21) to yield only 16 per cent of the absorption at 305 mμ as that given by an equivalent amount of glutathione. However, Binkley and Nakamura (5) report that they were unable to detect γ-glutamylcysteine among the breakdown products formed from glutathione in the presence of rat kidney preparations. If glutathione is cleaved in this fashion, then the enzyme responsible is present in rat kidney but not in rat liver. We have found that no glutathione disappearance occurs in the presence of rat liver homogenate within the pH range 5.0 to 8.1 as measured by the alloxan method.

DISCUSSION

The manometric method described here provides a rapid procedure for following γ-glutamyl transpeptidase activity near its optimal pH with glutathione as the γ-glutamyl donor. The method has proved useful for the determination of the distribution of the enzyme in the tissues of various marine forms (1). It has also been used to follow enzyme activity during purification procedures and for the study of the reaction of various amino acids and related compounds with glutathione.[2] The method is, however, limited to use in the alkaline region and can be employed only when glutathione, or possibly γ-glutamylcysteine, is used as the γ-glutamyl donor. In this method the chelation of one of the products of the reaction, cysteinylglycine, may serve to drive the reaction forward, though no evidence for this has been obtained. The reasonable agreement in results obtained with this method and the alloxan method would suggest that any such effect is not a serious one during the initial stages of the reaction.

The nature of the compound formed between glutathione and alloxan which gives rise to the absorption of light maximally at 305 mμ in alkaline solutions is apparently not known (21). Since cysteinylglycine does not react to give a product absorbing in this region, it is apparent that absorption is dependent on the presence of the γ-glutamyl peptide linkage. In this connection it is of interest to note that alkaline solutions of either *S*-acetoacetyl-*N*-acetyl-thioethanolamine or the β-ketoacyl derivatives of coenzyme A give absorption spectra which yield a maximum in the region

[2] Revel, J. P., and Ball, E. G., unpublished results.

303 to 305 mμ (2, 19, 24). These observations indicate that the configuration —$CH_2 \cdot COCH_2 \cdot CO \cdot S \cdot CH_2CH_2 \cdot NH \cdot COCH_2$—, probably in an ionized enol form, can give rise to absorption in the region 303 to 305 mμ and suggest that a similar configuration is present in the reaction product formed from glutathione and alloxan.

Besides the quantitative methods described here, we have also investigated in this laboratory a procedure for the measurement of cysteinylglycine formed in the reaction by the use of the Sullivan and Hess method (25). We have not had, however, the success with the application of this reaction to the quantitative determination of γ-glutamyl transpeptidase activity that other workers appear to have had (4, 9). Hird and Springell (13) have reported the quantitative estimation of transpeptidase activity by the use of a modification of the Nakamura and Binkley (20) procedure in which cysteine yields a blue color when treated with brucine and persulfate. This method apparently depends upon the conversion of the cysteinylglycine first formed into cysteine by a dipeptidase present in crude kidney homogenates.

SUMMARY

A manometric method has been described for following the participation of glutathione in reactions catalyzed by γ-glutamyl transpeptidase which is based upon the uptake of carbon monoxide that occurs in the presence of ferrous salts by the cysteinylglycine released in the reaction. Data have been presented to show that enzyme activity is not affected by the conditions of the method. A comparison has been made of the results obtained by the manometric method with those obtained by measuring the disappearance of glutathione by the alloxan method of Patterson and Lazarow. The two methods give concordant results under a variety of conditions. The pH-activity curve of γ-glutamyl transpeptidase has been determined by the alloxan method for the hydrolysis of glutathione and for the transpeptidation of glutathione with (1) glycylglycine and (2) L-alanine. The position of the pH curve for transpeptidation is shown to vary with the pK value of the amino group of the γ-glutamyl acceptor in such a way as to indicate the participation of the uncharged form in the reaction.

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PURIFICATION AND PROPERTIES OF FRUCTOSE-1,6-DIPHOSPHATASE*

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In previous communications (1, 2), the activation of FDPase[1] by proteolytic enzymes and a purification of the enzyme after a preliminary autolysis, as used by Gomori (3), were reported. A purification without autolysis has now been made to enable a study of the nature of the proteolytic activation of FDPase and to secure a more stable preparation of the enzyme for investigation of its properties.

EXPERIMENTAL

General Methods—Inorganic phosphate was determined by the method of Fiske and Subbarow (4), hexoses by the method of Mokrasch (5), α-amino nitrogen by the method of Moore and Stein (6), and ammonia with Nessler's reagent. Protein was estimated by using 1.5 ml. samples with 6 ml. of a biuret reagent containing 230 ml. of 18 to 20 M NaOH, 2.5 gm. of $CuSO_4 \cdot 5H_2O$, and 50 ml. of 28 per cent ammonia per liter. The use of ammonia, suggested by Dr. R. J. Winzler, permits direct estimation of protein in samples containing ammonium sulfate. The method is useful with samples containing from 5 to 15 mg. of protein. The addition of 1 ml. of 4 M ammonium sulfate, which is a quantity greater than that added with any of the samples, increases the optical density by an amount corresponding to 0.8 mg. of protein. A correction should be made for this effect with samples containing more than 2 m.eq. of ammonium ion. The reagent is as sensitive as, and more stable than, the usual biuret reagents. In more dilute samples, protein was measured by the method of Lowry *et al.* (7). All the operations were conducted at 0–4° with cold material unless otherwise specified. Centrifugations were made at 3000 × *g*.

Purification of FDPase—Livers from white rabbits which had been fasted 12 to 24 hours were dispersed with a Waring blendor for 3 minutes in 4 ml. per gm. of tissue of 0.05 M sodium lactate, pH 4.00, containing 0.001 M cys-

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[1] The following abbreviations are used: FDP, D-fructose-1,6-diphosphate; FDPase, fructose-1,6-diphosphatase; EDTA, ethylenedinitrilotetraacetic acid; F6P, D-fructose-6-phosphate; and F1P, D-fructose-1-phosphate.

teine. The supernatant fluid (S_0) obtained by centrifugation for 30 minutes was filtered through glass wool and brought to 2.08 M ammonium sulfate, pH 8.50, by the addition of the solid salt and 15 M NH_4OH. Directions for calculating the molarity of the ammonium sulfate have been given previously (2). The pH was measured on an aliquot diluted to 0.2 M ammonium sulfate, and the amount of NH_4OH required may be estimated from the formula, $pH = 7.29 - \log M + \log n$, where n ml. of 28 per cent ammonia are added per liter of M molar ammonium sulfate, and the pH is measured at 25°.

After 15 minutes of slow stirring, the precipitate was removed by centrifugation for 15 minutes and the supernatant fluid (S_1) was raised to 2.65 M by the addition of 3.75 M ammonium sulfate, pH 8.50. After 20 minutes, the precipitated enzyme was collected by centrifugation for 20 minutes, drained, and redissolved in 0.15 ml. of water per ml. of S_0. The solution (R_2) was analyzed for ammonia, and the value was used to determine the ammonium sulfate concentration after correction for the NH_4OH added. The solution was then brought first to 1.95 M, pH 8.50, by addition of solid ammonium sulfate and 15 M NH_4OH, and then to 2.14 M with 3.75 M ammonium sulfate, pH 8.50. After 15 minutes of stirring, the precipitate was removed by centrifugation for 20 minutes and the supernatant fluid (S_3) brought to 2.59 M with 3.75 M ammonium sulfate, pH 8.50, stirred for 20 minutes, and centrifuged for 30 minutes. If the livers contained excessive glycogen, a gel-like matrix, which prevented adequate sedimentation, formed at this point and spoiled the preparation.

The precipitated enzyme was dissolved in 0.15 ml. of water per ml. of R_2, and the solution (R_4) was adjusted to pH 7.1 ± 0.1 with 50 per cent lactic acid. It was heated in a water bath maintained at 60° ± 0.5°, with brisk stirring, until 5 minutes after its temperature had risen to 58°, and was removed and chilled below 10° in ice. The pH was lowered to 5.1 ± 0.1 with 1 M lactic acid. 0.015 ml. of a solution containing 100 mg. per ml. of salmine sulfate was added for each ml. of R_4. The salmine sulfate (Krishell) was purified by three precipitations with alcohol. After 10 minutes of stirring, the precipitate was removed by centrifugation for 10 minutes. The supernatant fluid (S_5) was brought to pH 3.70 ± 0.02 with 50 per cent lactic acid, stirred slowly for 20 minutes, and then brought to pH 7.7 ± 0.2 by the addition of 6 M potassium carbonate. The amount of carbonate was previously calculated because too slow an addition caused undesirable foaming.

After removing a precipitate by 10 minutes of centrifugation, an aliquot of the supernatant fluid (S_6) was tested to determine the amount of 15 M NH_4OH required to bring the pH to 10.49 ± 0.02. This amount was added with vigorous stirring. Immediately after completion of the addition, the

vessel was transferred to an ice-salt bath at −20°. 1 minute after the addition of the base was completed, the previously calculated amount of 5 M H_2SO_4 at −20° which was necessary to bring the pH to 7.65 ± 0.15, was added with vigorous stirring. The pH was then adjusted to 7.0 ± 0.1 with 50 per cent lactic acid at 0° and the precipitate removed by centrifugation for 10 minutes.

The supernatant fluid (S_7) was analyzed for protein and for ammonia. 10 mg. of Whatman standard grade cellulose powder were added per mg. of protein, followed by solid ammonium sulfate to 3.0 M. Stirring was maintained for 15 minutes after solution of the salt and the slurry was poured onto a cellulose column with a bed 20 cm. long and of such diameter that there was 1 ml. of bed volume per mg. of protein. If necessary, the amount of protein could be increased at least three times, but the cellulose added with the protein was increased proportionately. The cellulose column was prepared as follows.

A slurry of cellulose powder in 0.5 M ammonium sulfate containing 0.001 M EDTA was packed in portions in the tube by application of 0.5 atmosphere of air pressure at the top until the depth was 20 cm. after the bed ceased to contract. Air pockets in the column cannot be tolerated. In the cold, 0.5 bed volume of 3.0 M ammonium sulfate containing 0.001 M EDTA was forced through the column until the surface of the bed was just exposed and firm. The enzyme sample was then added, the bed depth being increased approximately 1 cm.

After the addition of the enzyme, the free liquid was driven into the column until the surface was exposed and a tightly fitting disk of filter paper was tamped on the surface, care being taken to eliminate air beneath it. The column was connected to a mixing flask containing 6 bed volumes of a stirred solution of 2.75 M ammonium sulfate, 0.001 M EDTA, and 0.001 per cent Pluronic L44.[2] The mixing flask was connected to a reservoir of 0.001 M EDTA containing 0.005 per cent Pluronic L44. The chelating and surface-active agents serve to sharpen the fractions. Air pressure was applied to the reservoir and adjusted as necessary to maintain a flow of 5A ml. per sq. cm. per hour, where A is the cross sectional area of the column. The effluent was collected in 0.05 bed volume fractions.

The fractions were analyzed for protein, and the largest peak, which contained the enzyme, was assayed for FDPase. The fractions included in the 80 per cent of the eluted enzyme with the highest specific activity were pooled, and this eluate (E_0) was analyzed for ammonia. Typical elutions from this and the following column are presented in Fig. 1.

E_0 was then passed through a second cellulose column prepared and op-

[2] Pluronic L44 is a non-ionic surface-active agent prepared from propylene glycol and ethylene oxide by the Wyandotte Chemicals Corporation.

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erated as was the first, except that its size was adjusted to the lesser amount of protein and all the solutions, including the enzyme-cellulose slurry, were adjusted to pH 7.5. The pooled fractions containing 80 per cent of the eluted enzyme with the highest specific activity were concentrated for storage by bringing the solution to 3.0 M ammonium sulfate with the solid salt,

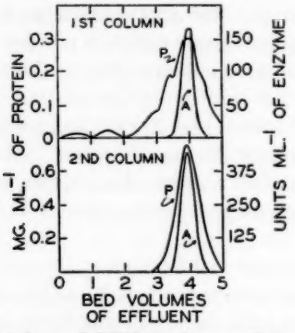

FIG. 1. Elution of protein and FDPase from cellulose columns. *A* indicates enzyme, and *P* protein.

TABLE I

Purification of Fructose-1,6-diphosphatase

Fraction No.	Specific activity	Total activity	Recovery
	units per mg. protein	*units*	*per cent*
S_0	1.8	14,500	100
R_3	15	12,000	85
R_4	25	8,000	57
S_5	31	7,200	50
S_6	42	6,800	47
S_7	95	6,100	42
E_0	400	5,800	40
E_1	570	4,900	32

collecting the precipitate by centrifugation, and dissolving it in water to give a protein concentration of 30 to 50 mg. per ml. The solution (E_0) was stored at −20°. A summary of a typical purification is given in Table I. The highest specific activity obtained by this procedure was 720 units per mg. of protein.

Attempts at crystallization of the enzyme failed even though the preparations were nearly homogeneous both in behavior on elution and in electrophoretic mobility. Attempts were made with ammonium sulfate at pH 5.5, 7.5, or 8.5, with ammonium sulfate containing 0.005 M FDP, Mg^{++},

Mn^{++}, Hg^{++}, or cysteine at pH 5.5 or 8.5, with magnesium chloride, and with magnesium acetate or ammonium acetate as precipitating agents. The last three salts would not precipitate the enzyme. Phosphatases as a class appear to resist crystallization.

Assay—The assay of FDPase was conducted as previously described (1, 2), except that serine buffer, pH 9.40, was used. Final concentrations of 0.0025 M magnesium sulfate, 0.0005 M manganous sulfate, and 0.003 M cysteine gave maximal activity through the S_7 stage of purification, and 0.0005 M magnesium sulfate, 0.0005 M manganous sulfate, and 0.003 M cysteine gave maximal activity with purer fractions. The FDP used in the assay was prepared from the cyclohexylammonium salt (8) with a sodium Dowex 50 column. The mixture of the three activators, the buffer, and the enzyme was incubated for 5 minutes at 0° before the reaction was started

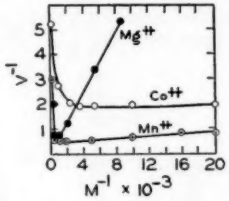

FIG. 2. Lineweaver-Burk plot of effect of metal ions on FDP hydrolysis by FDPase. The velocity of the enzymatic reaction was determined in the presence of varying concentrations of the metallic ions shown.

by the addition of FDP. 1 unit of enzyme (2) releases 1 μmole per hour of inorganic phosphate from FDP under the assay conditions.

Properties of FDPase—The stability of the enzyme with changing pH values was determined at 50°. After incubation for 30 minutes in 0.05 M buffers as indicated, over 90 per cent of the activity remained in acetate, pH 4.7, or glycine, pH 10.0, but less than 10 per cent in lactate, pH 3.1, or lysine, pH 11.4.

The enzyme had maximal FDPase activity at pH 9.5 under the assay conditions, as did the autolyzed enzyme (2, 3), and had approximately 30 per cent of maximal activity at pH 7.0 or 11.0. The activity fell to zero at pH 4.2 or 11.4, the upper limit apparently being defined by the increasing instability of the enzyme.

The effect of metal activators is summarized in Fig. 2. Although 0.001 M Mn^{++} alone was as effective as was the activator mixture used in the assay, this was not true with the autolyzed enzyme (2), and the mixture provided a safety factor. Owing to competition, the optimal concentration of each metal in a mixture was less than its optimum when used alone.

The action of some inhibitors is summarized in Table II. The reversal by cysteine of the inhibition by sulfhydryl reagents was not affected by the order of addition. The inhibition by F6P, but not by F1P or inorganic phosphate, suggests that the greater affinity of the enzyme is for the 6-phosphate position of FDP. The accumulation of the end-product of the enzymatic reaction could exert only a slight control on the physiological function of the enzyme. Fluoride was without effect when Mn^{++} was included in the medium. The absence of any effect by cyanide invalidates the proposal of Gomori (3) for distinguishing FDPase from other alkaline phosphatases with this reagent.

TABLE II

Effect of Inhibitors on Fructose-1,6-diphosphatase

The concentration of both Mg^{++} and Mn^{++} was 0.0005 M when used together. Mg^{++} was 0.0015 M and Mn^{++} 0.001 M when used singly. Cysteine was 0.003 M except where noted.

Inhibitor	Inhibition	Cofactors
	per cent	
0.005 M NaCn	0.0	Mg or Mn
0.005 " NaF	0.0	" + " + cysteine
0.005 " "	50	"
0.0001 M NaF	23	"
0.0001 N I_2	100	" or "
0.0001 " "	98	" + " + cysteine
0.005 M *p*-chloromercuribenzoate	100	" or "
0.005 " "	94	" + " + cysteine
0.0001 M "	63	" or "
0.0001 " "	11	" + " + cysteine
0.001 M $HgCl_2$	100	" or "
0.001 " "	30	" + " + 0.012 M cysteine
0.10 M fructose	5	" + " + cysteine
0.10 " glucose	0.0	" + " + "
0.005 M F1P	0.0	" + " + "
0.005 " F6P	22	" + " + "
0.010 " Na_2HPO_4	0.0	" + " + "
0.010 " cystine	0.0	" or Mn
0.005 " mucic acid	5	" + " + cysteine

Proteolytic Activation—Twice crystallized papain (Worthington Biochemicals), incubated with FDPase according to the conditions of Kimmel and Smith (9), caused an increase in the activity of the phosphatase. The changes are summarized in Fig. 3. If the papain or its EDTA-cysteine activator was omitted, no measurable change in α-amino nitrogen or protein occurred, and the activity of the FDPase declined 2 to 3 per cent during

the incubation. For comparison, autolyzed FDPase was prepared (2), and electrophoretic analyses of the autolyzed enzyme, the non-autolyzed

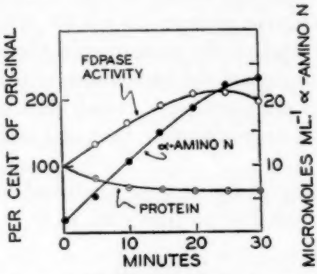

FIG. 3. Effect of papain on FDPase. 0.04 mg. of papain was incubated with 2.2 mg. of FDPase per ml. of a medium containing 0.001 M EDTA, 0.005 M cysteine, and 0.05 M citrate, pH 5.5, at 38°. Aliquots were removed at various times and the FDPase activity was determined.

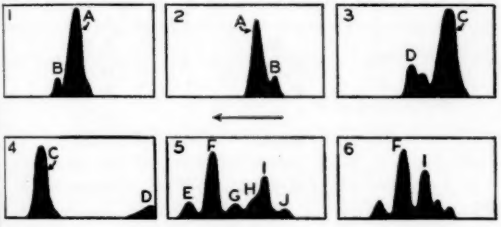

FIG. 4

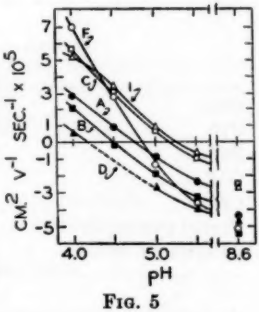

FIG. 5

FIG. 4. Electrophoretic patterns of FDPase preparations. *1*, non-autolyzed preparation at pH 8.60; *2*, non-autolyzed preparation at pH 4.00; *3*, non-autolyzed preparation at pH 8.60 after treatment with papain; *4*, non-autolyzed preparation at pH 4.00 after treatment with papain; *5*, autolyzed preparation at pH 8.60; *6*, autolyzed preparation at pH 4.00. The starting boundary is at the right margin of each diagram.

FIG. 5. Variation in electrophoretic mobility of components of FDPase. The components are those designated by the same letters in Fig. 4.

enzyme, and the non-autolyzed enzyme treated with papain were made in the Antweiler microelectrophoretic apparatus at 3.8° ± 0.1°. 1.5 to 2.0 mg. samples of enzyme were dialyzed in the special cells of the apparatus for at least 5 hours against 2000 volumes of a buffer containing 0.1 M glucose and then diluted to 0.25 ml. with the buffer. The buffer was ammonium acetate, ionic strength 0.10 at pH 8.60, and 0.05 at lower pH values.

Analyses were made by interferometry. The results are presented in Fig. 4 and the variation of mobility with pH is shown in Fig. 5 for the various components designated by the letters in Fig. 4. Two minor components of the non-autolyzed enzyme appeared at pH 8.6, with the major peak constituting 88 to 90 per cent of the pattern area throughout the pH range. Treatment with papain caused the appearance of other components and a shift in the mobility of the main peak, which then had a mobility and an isoelectric point closely approximating that of Component I of the autolyzed preparation.

The relative specificity of the enzyme is altered by papain (Table III). The ratio of activity with F1P to that with FDP is six times higher with

TABLE III

Specificity of Fructose-1,6-diphosphatase

The activity of a non-autolyzed FDPase preparation before and after treatment with papain under the conditions described in Fig. 3 is shown as the fraction of the activity of the untreated enzyme on FDP. All the substrates were 0.005 M.

Substrate	Relative hydrolysis	
	Before papain	After papain
FDP	1.00	1.95
F1P	0.06	0.05
F6P	0.00	0.00
G1P	0.00	0.00
G6P	0.00	0.00
Phenyl phosphate	0.00	0.00
β-Glycerophosphate	0.00	0.00

the non-autolyzed preparation than it is with the autolyzed enzyme (2), and the ratio is lowered by the treatment with papain.

DISCUSSION

The product of FDPase action has been shown to be F6P (10, 11). Some preparations of the autolyzed enzyme acted on FDP to yield products with high acid-labile phosphate (2), but this effect has not been encountered since and must be ascribed to occasional accidental concentration of other enzymes reacting with FDP or F6P.

The point of primary interest is the change of activity in the enzyme caused by proteolysis, and the possible physiological rôle of this effect will be the subject of a later communication. The unfortunate polydispersity of the autolyzed enzyme makes it difficult to decide which peak represents FDPase. The similar properties of the main peak of the non-autolyzed preparation and of Component I of the autolyzed enzyme tempt a sug-

gestion that they are the same, but the data do not stand as proof of the suggestion. In addition to the data presented to indicate the changes in the properties of FDPase by autolysis or treatment with papain, it is also noteworthy that alumina Cγ adsorbs non-autolyzed FDPase only slightly, whereas it readily adsorbed the autolyzed enzyme (2).

The data indicate that FDP is bound to the enzyme most strongly by its 6-phosphate group, with the lesser importance of the carbon skeleton suggested by the lower affinity and activity of F1P. The proteolytic increase in activity toward FDP but not toward F1P may indicate an alteration in the binding of the 6-phosphate or the uncovering of a second active site of more rigid specificity.

If the most active FDPase preparation obtained, with a specific activity of 720 units $mg.^{-1}$, was treated with papain and the expected doubling of the enzymatic activity and halving of the protein concentration were obtained (Fig. 3), the resultant enzyme would cleave over 4000 moles of FDP per 100,000 gm. of protein per minute.

SUMMARY

A purification of fructose-1,6-diphosphatase without autolysis has been made. The properties of the enzyme, including the alteration in its properties and activity by the action of papain, are reported.

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A STUDY OF TWO YEAST PROTEINASES*

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Autolysis is a procedure frequently employed in the isolation of materials from yeast. Although the intracellular yeast proteinases play an important rôle in autolysis, these enzymes have not been characterized by using up to date methods of analysis, and the existing literature concerning yeast proteinases presents conflicting results. In 1917, Dernby (1) reported that yeast contains two proteinases, a "pepsin" and a "tryptase." The presence of two proteinases was indicated mainly by the fact that a yeast autolysate showed two pH optima for gelatin liquefaction; namely, at pH 4.5 and pH 7.0. In 1926–28, Grassmann, Willstätter, and coworkers published a series of papers on the proteolytic activity of yeast (2–4). These authors concluded that there is only one proteinase in yeast. This enzyme was reputed to be of the papain type with an optimal pH of 5.0 as measured by an alcoholic KOH titration of gelatin hydrolysis (4). However, in 1936, Hecht and Civin (5) claimed that yeast contains a "pepsin" which displays optimal activity at pH 1.8.

In the present paper, the separation and partial characterization of two individual yeast proteinases are described. One has an optimal pH of about 3.7 and is rapidly denatured by urea. The other has optimal activity at pH 6.2 and may be activated by concentrated urea solutions.

EXPERIMENTAL

Most of the analyses for proteinase activity were made according to the method of Anson (6) with slight modification. Both acid-denatured hemoglobin and alkaline urea-denatured hemoglobin substrates were employed. The former substrates contained 2 per cent hemoglobin with 0.07 N citrate as a buffer. The latter contained 2 per cent hemoglobin, 6.6 M urea, 0.07 N acetate, 0.07 N phosphate, and 0.07 N borate as a universal buffer. 1 ml. of enzyme solution was added to 5 ml. of substrate and the mixture was incubated for 10 or 20 minutes at 30°. 10 ml. of 0.3 N trichloroacetic acid were added and the digestion products in the filtrate were measured either

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by reaction with the Folin-Ciocalteu reagent or by reading the absorption of the filtrate at 280 mμ. Both of these procedures for measuring the digestion products were employed in this investigation and gave similar results. Aliquots of crude yeast preparations were adjusted to the pH of the substrate immediately before analysis.

Protein concentrations were measured by the method of Robinson and Hogden (7), twice recrystallized pepsin being used as a reference protein.

Results

Autolysis and Purification—The procedure employed for extraction and partial purification of the yeast proteinases is outlined in Table I. The best yields of these enzymes were obtained when the initial autolysis was carried out at pH 7.0 to 7.6. Toluene, dioxane, and glycerol were also used to plasmolyze the yeast, but the subsequent autolysate showed little or no proteinase activity. Ethyl acetate autolysates yielded only about 50 per cent of the activity obtained with chloroform or ether. The majority of proteinase A present in the whole autolysate is in an insoluble condition and does not appear in the supernatant liquid (Step 2).

The pH-activity curve of the supernatant liquid obtained in Step 2 can be seen in the lower curves of Fig. 1. Curve A is the pH-activity curve obtained by using hemoglobin substrates containing no urea. Curve B represents the activity of the autolysate against urea-denatured hemoglobin. Evidence presented below indicates that Curves A and B are primarily manifestations of two different proteinases which, for the sake of brevity, will be referred to as proteinases A and B, respectively.

There are several peculiarities about Curves A and B which should be noted. (1) It was found that proteinase A is almost instantaneously destroyed by 5.5 M urea at pH 2.0 to 4.0. Therefore, this enzyme has little or no effect on the urea-containing substrates in this pH region; (2) the decline of Curve A above pH 3.8 is caused by the fact that the hemoglobin is not in a denatured form in the absence of urea in the higher pH range (8). Although there is considerable yeast proteinase activity against denatured hemoglobin in the region of pH 6 to 9, native hemoglobin is resistant to proteolysis in this range; (3) the peak at pH 5 in Curve B is caused by a rapid activation of proteinase B by the 5.5 M urea present in the hydrolysis mixture during analysis. In the following section, the fact that urea activates proteinase B at pH 5, but not at pH 4 or 6, is illustrated.

Similar pH-activity curves were displayed by autolysates of four different strains of *Saccharomyces cerevisiae:* two of bakers' yeast grown in pure culture[1] and a top and a bottom brewers' yeast. Similar curves were also

[1] Kindly supplied by Dr. A. S. Schultz of these Laboratories.

TABLE I

Purification of Yeast Proteinases

800 gm. of compressed Fleischmann's bakers' yeast were plasmolyzed with 400 ml. of chloroform or ether. After 30 to 60 minutes, 1.2 liters of water were added and the pH was adjusted to 7.0 with NaOH. (No proteinase activity was detectable at this stage.) After autolysis for 18 hours at 25°, the suspension was centrifuged and the supernatant solution adjusted to pH 5.0 and allowed to stand under toluene for 18 hours at 25°. 2 volumes of 95 per cent ethanol were added gradually at about 10°, and the resulting precipitate was dried *in vacuo* over $CaCl_2$. The activities are expressed in terms of the micrograms of "tyrosine" produced per 5 ml. of trichloroacetic acid filtrate after a 10 minute digestion period at 30°. Proteinase A was measured by using acid-denatured hemoglobin at pH 3.5; proteinase B was measured by using urea-hemoglobin substrates at pH 6.2.

Step No.		Proteinase A activity		Proteinase B activity	
		per ml.	*per mg. protein*	*per ml.*	*per mg. protein*
1	Entire chloroform autolysate....	685		168	
2	Supernatant liquid from autolysate.....	176	39	168	37
3	Supernatant after incubation at pH 5.....	350	350	520	520
4	63% ethanol ppt.....		365		790

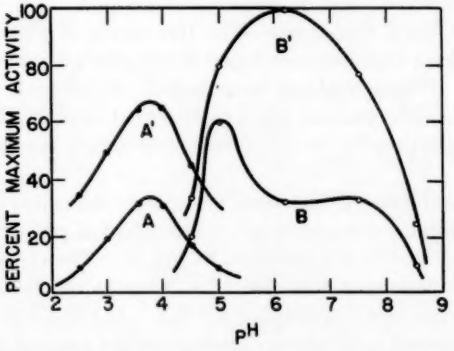

FIG. 1. Change in the pH-activity curves of yeast proteinases which occurred when supernatant liquid of a crude bakers' yeast autolysate was incubated at pH 5 for 18 hours at 25°. The substrates employed for Curves A contained no urea, whereas those used for Curves B contained 6.6 M urea. The lower curves (A and B) represent the activity of the autolysate before incubation; the upper curves (A' and B') represent the activity of the same autolysate after incubation.

obtained with an autolysate of a strain of *Saccharomyces mangini*. The remainder of the data reported here was obtained by using autolysates of Fleischmann's compressed yeast for bakers.

In Table I, when the supernatant liquid from the autolysate was adjusted to pH 4.5 to 5.0, a heavy precipitate formed and much of the proteinase activity was found in the precipitate. The enzymes are merely adsorbed on the inert precipitate, since purified proteinase preparations are completely soluble at pH 4.5 to 5.0. This precipitate is not removed and usually disappears gradually during incubation at pH 5. During overnight incubation at pH 5, a remarkable self-purification of the proteinases takes place. An activation occurs at this pH and the total amount of proteinases A and B activity increases about 3- to 4-fold. In addition, it has been found that the proteinases digest the inert proteins of the autolysate most rapidly at pH 5. Thus the activity per mg. of protein increases 10- to 20-fold. Further purification has been obtained by dialysis and by alcohol, acetone, or ammonium sulfate precipitation of the activated autolysate. The proteinases may be precipitated from the supernatant liquid of the autolysate with 63 per cent ethanol before activation to obtain a stable dry preparation which may be activated subsequently by incubation at pH 5.

The specific activity of proteinase B in the alcohol precipitate (Table I) is about 5 per cent of that of crystalline trypsin. The amount of activated proteinase B which is obtainable from 1 gm. of compressed bakers' yeast is equivalent to about 0.1 mg. of trypsin.

Activation of Yeast Proteinases—The top curves of Fig. 1 illustrate the pH-activity curves of proteinases A and B after the activation at pH 5 mentioned above. The optimal pH for activated proteinase B is now approximately pH 6.2. The peak at pH 5 disappeared because this enzyme was activated and the urea in the pH 5 substrate could not cause additional activation.

The rate of activation of the yeast proteinases depended upon the pH at which the autolysate was incubated. The effect of pH on the activation of proteinases A and B is illustrated in Fig. 2. The optimal pH for the activation of proteinase A was about 4.9, whereas the optimal pH for the activation of proteinase B was about pH 4.4. At pH 3.8 to 4.3, proteinase B could be activated without any increase in the amount of proteinase A activity.

The rate of activation of proteinase B was increased by the presence of calcium ions. In an experiment in which an unactivated preparation was incubated at pH 5, the presence of 1 per cent $CaCl_2$ not only increased the activation rate but also increased greatly the total amount of proteinase B formed. Calcium ions are known to have a similar effect on the conversion

of trypsinogen to trypsin (9). The rate of activation of proteinase B was not increased by 0.1 M cyanide or 0.0004 M cysteine.

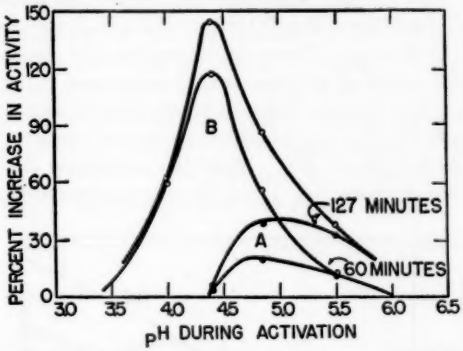

FIG. 2. The extent of activation of the yeast proteinases as a function of pH. Four aliquots of an unactivated autolysate were incubated at four different pH values at 30°. After 60 and after 127 minutes, each aliquot was analyzed for proteinase A and proteinase B activity, acid-denatured hemoglobin at pH 3.5 being used as substrate for proteinase A, and urea-denatured hemoglobin at pH 6.2 as substrate for proteinase B.

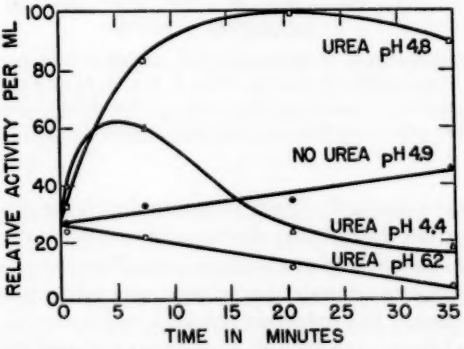

FIG. 3. The effect of 3 M urea on the proteinase B activity per ml. of an unactivated yeast autolysate. Aliquots of the autolysate were incubated at 30° and the proteinase B activity was measured at intervals by withdrawing 1 ml. aliquots and incubating for 10 minutes with 5 ml. of the urea-hemoglobin substrate at pH 6.2.

It was found that concentrated urea solutions greatly accelerated the rate of activation of proteinase B at pH 5 (Fig. 3). The maximal activation in a 20 minute period was obtained with 3 M urea at pH 4.8. Proteinase B was also rapidly activated at pH 4.4 by 3 M urea. However, the

destruction by urea was faster at this pH and prevented the attainment of greater activation. At pH 6.2 there was no apparent activation, only gradual destruction by urea. Urea concentrations from 1.0 M to 6.0 M were found to activate proteinase B in 30 minutes at pH 5, the maximal activation occurring in 3 M urea. These results explain the sharp peak at pH 5 in the pH-activity curve of an unactivated proteinase B preparation.

Enzyme Calibration Curves—When activated proteinase B was exposed to 5.5 M urea at pH 5.0, 6.2, and 7.5, the enzyme was found to be inactivated appreciably in 20 minutes at 30°. Since the proteinase B substrates contain 5.5 M urea during digestion, it was therefore expected that the enzyme

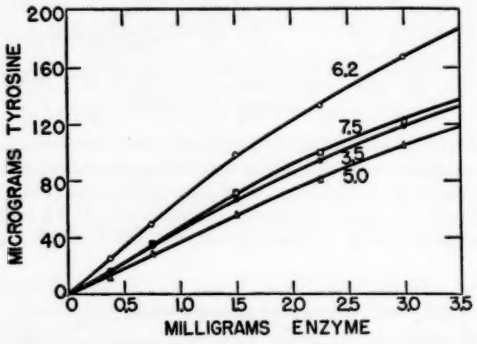

FIG. 4. Calibration curves of activated yeast proteinases at various pH values. An alcohol precipitate, similar to that in Table I, Step 4, of an activated autolysate was used. The activity of proteinase A was measured with acid-denatured hemoglobin at pH 3.5. For proteinase B, urea-denatured hemoglobin at pH 5.0, 6.2, and 7.5 was employed.

would be destroyed gradually during incubation with the substrate; in fact, digestion rate curves showed that the rate of hemoglobin decomposition at pH 5.0, 6.2, and 7.5 decreased gradually during a 20 minute digestion period.

In order to measure accurately proteinase activities under these conditions, it was necessary to employ enzyme calibration curves. Fig. 4 shows the relationship between the amount of enzyme employed and the digestion products from the action of proteinase A at pH 3.5 and proteinase B at pH 5.0, 6.2, and 7.5. The extent of digestion was proportional to the enzyme concentration only at the lower enzyme levels. Therefore, in order to obtain a true measure of the amount of proteinase in a given preparation, calibration curves were employed throughout this work in the manner described by Anson (6).

Effect of Sulfhydryl Reagents on Yeast Proteinases—*p*-Chloromercuribenzoate (pCMB), which is a specific reagent for sulfhydryl groups (10),

has been found to inactivate yeast proteinase B, but to have no effect upon proteinase A (Fig. 5). Curves similar to those in Fig. 5 were also obtained when higher levels of pCMB were employed, and when the exposure to this reagent was carried out at other pH values between pH 4.5 and 7.0. The residual activity against urea-containing substrates between pH 5 and 7 may be attributable to proteinase A, which may be more resistant to the urea in the substrates in this pH range, although it was inactivated immediately by urea at lower pH values. A preparation of proteinase B containing no proteinase A was found to be inactivated completely by pCMB. The activity removed by this reagent may be restored by the addition of NaCN.

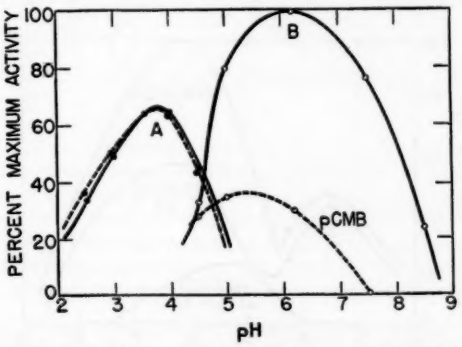

FIG. 5. The effect of *p*-chloromercuribenzoate on yeast proteinases. The solid lines represent the pH-activity curves of an activated preparation of proteinases A and B. After incubation in a solution containing 0.001 M *p*-chloromercuribenzoate at pH 5 for 60 minutes at 25°, the preparation showed pH-activity curves represented by the broken lines.

Although proteinase B appears to contain essential SH groups, these groups differ from those of papain. Cyanide or cysteine must be present; otherwise papain cannot attack hemoglobin (6). These compounds were without effect on the splitting of hemoglobin by yeast proteinases. In addition, iodoacetate, which inactivates papain, had no effect on the yeast proteinases.

It was found that 0.15 mg. per ml. of purified ovomucoid (Worthington Biochemical Laboratories) had no effect on proteinase A, but caused a 40 per cent inhibition of proteinase B. Crude soy bean extracts which were active against trypsin had no effect on either yeast proteinase.

Thermostability of Yeast Proteinases—The effect of pH on the stability of yeast proteinases at 50° was studied. Distinct pH-stability curves were obtained; proteinase A was most stable at pH 4.8, while proteinase B was most stable around pH 5.7. Fig. 6 represents the effect of heating aliquots

of an activated preparation at pH 4.0 and at pH 7.1 on the pH-activity curves of the yeast proteinases. The aliquot heated at pH 7.1 was entirely freed of proteinase A, while retaining about half of proteinase B. When this heated aliquot was treated with pCMB, it was completely inactivated. The aliquot heated at pH 4.0 retained most of its proteinase A activity and a small residual activity against urea-hemoglobin substrates at pH 5 to 7. The pH-activity curve of this aliquot was unaffected by pCMB. Thus, the effect of heating at pH 4 was similar to the effect of pCMB on the yeast proteinases.

Action on Other Proteins—pH-activity curves for the splitting of other proteins by unactivated and by activated preparations of yeast proteinases

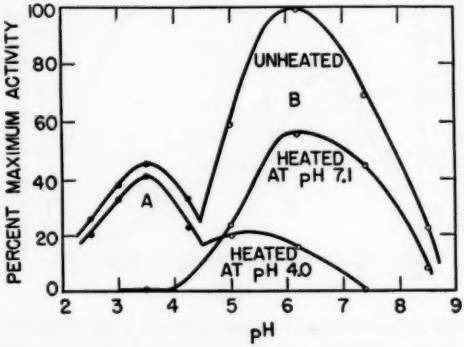

FIG. 6. pH-activity curves of an activated yeast proteinase preparation before and after heating aliquots at pH 4.0 and at pH 7.1 for 20 minutes at 50°. ●, activity against acid-denatured hemoglobin; ○, activity against urea-denatured hemoglobin.

were determined. The methods employed were identical to those used with hemoglobin. The pH-activity curves of unactivated and activated preparations with urea-denatured ovalbumin or pumpkin-seed globulin (Difco) as substrate were similar to the curves obtained with hemoglobin (Fig. 1). The splitting of twice crystallized ovalbumin by unactivated yeast proteinases showed an additional peak at pH 7.8 (Fig. 7). It is possible that this peak represents the activity of a third yeast proteinase. Native ovalbumin was not attacked at any pH between 2 and 9, except for a slight hydrolysis in the region of pH 3.0, presumably attributable to proteinase A.

When urea-denatured lysozyme, fibrinogen (Armour), or casein was employed as substrate, unactivated yeast preparations did not show any peak at pH 7 to 8 in the pH-activity curves (Fig. 7). A curve (not shown) was determined by using fibrinogen substrates containing no urea. The activ-

ity increased steadily from pH 4 to 9, reflecting only the increased denaturation and vulnerability of the fibrinogen at higher pH values.

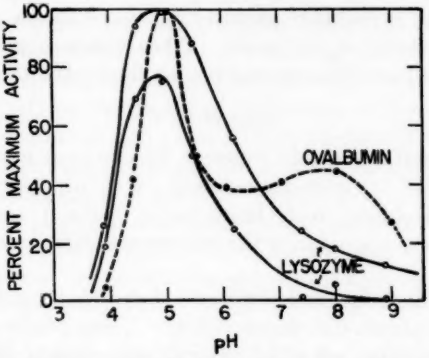

FIG. 7. Broken line, ovalbumin hydrolysis by an unactivated yeast proteinase preparation as a function of pH. The substrates were similar to the urea-hemoglobin substrates, containing 2 per cent recrystallized ovalbumin (11) and 6.6 M urea. Solid lines, hydrolysis of urea-denatured lysozyme by a yeast autolysate as a function of pH. The lower curve was obtained with an unactivated preparation and the upper curve with the same preparation after partial activation. The substrates contained 6.6 M urea and 2 per cent lysozyme prepared by adsorption on bentonite (12).

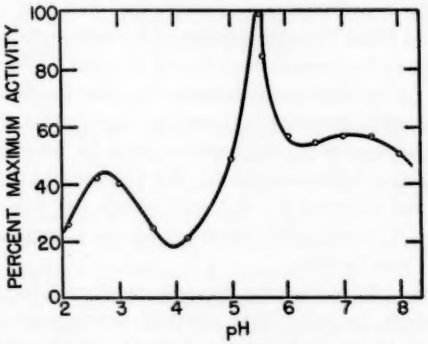

FIG. 8. The hydrolysis of casein by an activated yeast proteinase preparation as a function of pH. These substrates contained 2 per cent Difco isoelectric casein and no urea. Varying amounts of casein were insoluble in the range of pH 2.0 to 5.6.

When casein substrates containing no urea were employed, the pH-activity curve in Fig. 8 was obtained. Although this curve applies to an activated preparation, it is similar to the pH-activity curves for an unactivated preparation with hemoglobin substrates (Fig. 1). When the enzyme

solution used in Fig. 8 was treated with pCMB, the activity below pH 4 was unaffected, while the activity above pH 5 was practically abolished. The shape of the curve in Fig. 8 has a limited significance, however, because the casein digestibility is probably partially influenced by the effect of pH on the degree of solubility of the casein. When casein substrates were used, the peaks at pH 2.7 and 5.5 increased greatly during the activation process.

DISCUSSION

Proteinases A and B probably represent the two proteinases described by Dernby (1) in 1917. A preparation made by Dernby's procedure gave a pH-activity curve similar to the lower curves of Fig. 1, and this preparation underwent activation during the subsequent dialysis step employed by Dernby.

Grassmann, Willstätter, and coworkers stated that yeast contains only one proteinase, a papain-like enzyme (2–4). Using gelatin as a substrate, they reported an optimal pH of 5.0, optimal activation at pH 7, rapid activation by cyanide, and relative instability at pH 5.0. None of these properties agrees with the findings reported here. Grassmann *et al.* used an alcoholic KOH titration procedure to measure the gelatin hydrolysis; this method is a measure of the combined activity of proteinases and peptidases. It appears likely that the preparations used by Grassmann *et al.* contained peptidase activity, and some of the properties attributed to proteinase action were actually manifestations of peptidase activity. These authors state that they had freed their proteinase of known peptidases. However, additional peptidases have since been found in yeast (13).

As pointed out by Northrop and Kunitz (14), the alcoholic KOH method of proteinase analysis is insensitive as well as non-specific. Because of this insensitivity, Grassmann *et al.* employed a 24 or 48 hour digestion period at 40° in the analysis of their samples. We have found rapid inactivation of proteinases A and B below pH 3.5 and above pH 6.5 at 40°. Therefore, the optimal pH found under these conditions is primarily a reflection of the optimal pH for stability.

Hecht and Civin (5), employing Anson's pepsin substrate, claimed the discovery of a yeast "pepsin" with optimal activity at pH 1.8. These authors added a concentrated yeast autolysate to the unbuffered hemoglobin-HCl solution at pH 1.8. When this procedure was repeated here, it was found that the actual pH during hydrolysis was pH 3.0 to 4.0, which is approximately optimal for proteinase A activity. When the pH during digestion was controlled carefully, autolysates prepared by the method of Hecht and Civin displayed normal pH-activity curves similar to those in Fig. 1. They also reported that dilution or dialysis of an autolysate inactivated yeast "pepsin." These findings are explained by the fact that diluted

or dialyzed autolysates have less effect on the pH of the pepsin substrate; the actual pH during hydrolysis would be closer to pH 1.8 and therefore less proteinase A activity would be manifested.

None of the substrates employed in this investigation yielded continuous pH-activity curves which represented a valid measure of the effect of pH on hydrolysis rate. Native proteins are unsatisfactory substrates because of the effect of pH on the degree of insolubility or denaturation. Urea-containing substrates are also unsatisfactory because of the marked effect of urea in the destruction of proteinase A at pH values below 4.0 and the activation of proteinase B at pH 5.0. Nevertheless, all of the urea-denatured proteins employed as substrates showed approximately the same optimal pH for activated proteinase B. Since the isoelectric points of these proteins varied from 4.6 to 10.5, the optimal pH appears to depend on the nature of the enzyme rather than on the charge on the substrate molecule (15).

The effect of concentrated urea solutions on the activation of proteinase B is an unusual phenomenon. It may be caused by acceleration of the conversion of a zymogen to the active enzyme or by denaturation of an inhibitor present before activation. A similar activation, the conversion of protyrosinase to tyrosinase by 2 M to 7 M urea, has been reported by Bodine and Allen (16).

The author wishes to express his appreciation to Dr. Sutton Redfern for the helpful interest shown in this investigation.

SUMMARY

Two different proteinases have been obtained from bakers' yeast and have been partially characterized. Proteinase A is extremely labile in urea solutions and has an optimal pH of 3.7 for the splitting of acid-denatured hemoglobin. Proteinase B exhibits an optimal pH of 6.2, urea-denatured proteins being used as substrates. Proteinase B contains essential sulfhydryl groups, whereas proteinase A does not.

These enzymes are liberated simultaneously from the yeast cell by chloroform or ether autolysis. When the supernatant solution from this autolysate is incubated overnight at pH 5, the activity per ml. of proteinases A and B increases 300 to 400 per cent. During this process, these proteinases digest 70 to 80 per cent of the other proteins in the autolysate. At pH 4.3 proteinase B may be activated independently of proteinase A. The rate of activation of proteinase B is increased greatly by concentrated urea at pH 5. These proteinases have different pH-stability curves, so that they may be inactivated selectively by heat treatment.

The activity of the yeast proteinases in hydrolyzing various native and

denatured proteins was measured as a function of pH. Four different strains of *Saccharomyces cerevisiae* were examined and all were found to contain these two proteinases. These findings are compared and correlated with those of previous investigators.

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THE CONVERSION OF TESTOSTERONE-3-C^{14} TO C^{14}-ESTRADIOL-17β BY HUMAN OVARIAN TISSUE*

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There is a considerable body of biological data linking testosterone and other 19-carbon steroids with the estrogens. Both ovaries and testes may, under certain circumstances, produce androgens and estrogens (1–4). The administration of testosterone and other androgens to human subjects leads to an increased urinary excretion of estrogenic material (5–10), which has been identified as estrone, estradiol, and estriol (11). These observations suggest a possible conversion of 19-carbon steroids to phenolic estrogens.

Studies on the biosynthesis of steroid hormones have indicated that cholesterol can serve as a precursor of progesterone (12) and the adrenal cortical steroids (13). There is, however, some evidence that cholesterol is not a necessary intermediate in these biological syntheses (14). The conversion of cholesterol to estrogenic steroids has not been demonstrated. Heard *et al.* isolated C^{14}-estrone from the urine of a pregnant mare to which $CH_3C^{14}OONa$ was administered intravenously (15), but found no C^{14} in the urinary estrone after cholesterol-4-C^{14} was given by the same route (16).

Recently Heard *et al.* have reported the excretion of C^{14}-labeled estrone by a pregnant mare to which C^{14}-labeled testosterone was administered (17).

This paper reports the results of an experiment *in vitro* carried out to study the possible biochemical conversion of testosterone to estrogenic steroids by human ovarian tissue.

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This is publication No. 874 of the Cancer Commission of Harvard University. A preliminary report of this investigation was presented at the meeting of the American Society of Biological Chemists at its Forty-sixth annual meeting at San Francisco, April 11–15, 1955 (*Federation Proc.*, **14**, 175 (1955)).

EXPERIMENTAL

Determination of Radioactivity—All samples for radioactivity analysis were plated directly in stainless steel counting dishes (1 inch in diameter, $\frac{5}{16}$ inch in depth). The counts were then determined in a Tracerlab SC-16 windowless flow counter operated in the Geiger region. All counts are at infinite thinness with no more than 1 mg. being plated in any instance.

Determination of Estrogens—The counter-current distributions were analyzed for estrogen by the method of Engel *et al.* (18), based on fluorescence in H_2SO_4. A Farrand photofluorometer was employed for the measurement of fluorescence.

Tissue Incubation—Both ovaries were removed surgically from a 34 year-old woman with cervical carcinoma. The operation took place near the middle of a menstrual cycle. Grossly the ovaries appeared normal, and one contained a well developed follicle.

The ovaries were sliced by hand to a thickness of about 0.5 mm. and added to the incubation medium in a 125 ml. flask. The medium consisted of 20 ml. of human plasma, to which had been added 90 mg. of glucose, 0.18 mmole of citric acid, 0.02 mmole of sodium fumarate, 500 units of chorionic gonadotropin (A. P. L.; Ayerst, McKenna and Harrison), 500 units of equine gonadotropin (Equinex; Ayerst, McKenna and Harrison), and 0.23 mg. of testosterone-3-C^{14} (total of 550,000 c.p.m.), previously dissolved in 0.1 ml. of propylene glycol. The flask was filled with O_2, closed, and incubated at 37.5° with shaking for 3 hours. At the end of the incubation period, the mixture was frozen and kept until processed.

Extraction and Preliminary Fractionation—The tissue and medium were thawed, and 2 mg. each of estrone, estradiol,[1] and estriol were added as carriers. 2 volumes of hot acetone were added, and the mixture was shell-frozen and dried from this state. The dry tissue was extracted for 4 hours in a Soxhlet extractor with chloroform.

The chloroform was removed *in vacuo*, and the extract was dissolved in 50 ml. each of pentane and 90 per cent methanol. These solvents were equilibrated, and the 90 per cent methanol layer was removed. The pentane was extracted three additional times with 50 ml. of 90 per cent methanol. The pentane and methanol fractions were evaporated *in vacuo*. The residue from the methanol fraction (335,000 c.p.m.) was dissolved in 50 ml. of toluene, and this solution was extracted four times with 12 ml. of 1 N NaOH and twice with 12 ml. of H_2O. The combined alkali and water extracts were adjusted to pH 8.5, and the mixture was extracted three times with 45 ml. of diethyl ether (18). The residue, after evaporation of the ether, contained 5600 c.p.m. The neutral material, after removal of the toluene, contained 262,000 c.p.m.

[1] Estradiol as used throughout this paper refers to estradiol-17β.

Counter-Current Distribution between Toluene and 1 N NaOH—The phenolic fraction obtained from the preliminary fractionation was subjected to an eight transfer counter-current distribution between toluene and 1 N NaOH, 20 ml. in each layer (19). Separatory funnels were used and the lower layer was transferred.

When eight transfers had been completed, the pH of all the lower layers was adjusted to 8.5 to 9.0. The two layers in each funnel were reequilibrated, thereby extracting phenolic material into the toluene. Each lower layer was then reextracted twice with 10 ml. of diethyl ether. The combined toluene and ether extracts from each funnel were washed once with 3 ml. of H_2O and then evaporated to dryness. Aliquots were removed from each fraction for counting and for analysis for estrogens.

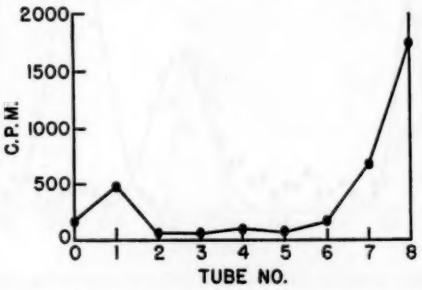

FIG. 1. Radioactivity of tubes from counter-current distribution of phenolic fraction. The upper layer is toluene; the lower layer, 1 N NaOH. Lower layers were transferred.

Fig. 1 shows the distribution of radioactivity in this counter-current distribution. There is a striking peak in activity in tube 8, indicating the presence of C^{14}-labeled phenolic material. Analysis for estrogens showed that they, too, were concentrated in tubes 5 to 8, with the peak in tube 8. When a sample of the testosterone-3-C^{14} used in this experiment was subjected to a similar eight transfer distribution, no peak in radioactivity was found in the higher numbered tubes.

99 Transfer Counter-Current Distribution—As the next step in the fractionation of the phenolic material, the contents of tubes 5 to 8 from the eight transfer distribution were pooled and subjected to a 99 transfer counter-current distribution in 30 per cent ethyl acetate-70 per cent cyclohexane with 50 per cent ethanol-50 per cent H_2O as the lower phase. The distribution was carried out in a 100 tube Craig machine (H. O. Post Scientific Instrument Company). All fractions from the distribution were evaporated *in vacuo* from the frozen state.

Aliquots were taken from selected tubes throughout the distribution for

determination of radioactivity and for analysis for estrogen. The results of these analyses are seen in Fig. 2. The scales of the ordinate for radioactivity and the ordinate for weight of estrogen were selected so that the peak in radioactivity in the estradiol region was the same height as the peak in weight.

This counter-current distribution shows that there is no significant C^{14} in the estrone region (E_1). There is, however, a peak in radioactivity coincident with the peak in weight of estradiol (E_2). The experimental values

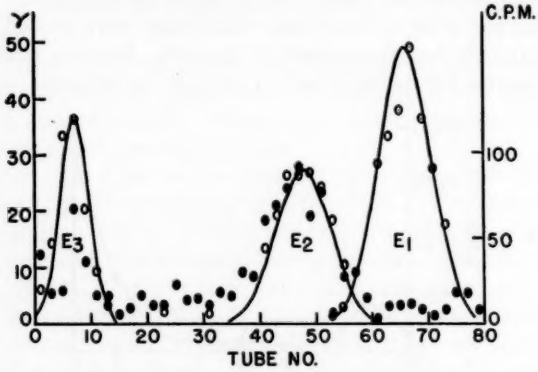

FIG. 2. 99 transfer counter-current distribution separating the three estrogens. Upper layer is 30 per cent ethyl acetate-70 per cent cyclohexane; lower layer, 50 per cent aqueous ethanol. Upper layers were transferred. In Figs. 2, 3, and 4, the following symbols are used: K, the partition coefficient (ratio of concentration in upper layer to concentration in lower layer); ○, experimental points for weight of estrogen referred to the ordinate on the left; ●, counts per minute referred to the ordinate on the right; solid lines, the best calculated theoretical curves through the experimental points for weight. E_1 = estrone, E_2 = estradiol-17β, E_3 = estriol. K for E_1 = 2.00, for E_2 0.94, for E_3 0.076.

for weight and for radioactivity fit a single theoretical distribution curve equally well, thus indicating that the estradiol contained C^{14}. There is also a peak in radioactivity in the same tube as the peak in weight of estriol (E_3). The difference in the height of the weight and radioactivity peaks is a result of the arbitrary adjustment of the two ordinates. It appeared likely, therefore, that the estriol was also labeled.

Redistribution of Estriol—The contents of tubes 2 to 14 (estriol region) of the preceding counter-current distribution (Fig. 2) were pooled. 4 mg. of additional carrier estriol were added. The estriol was then subjected to a forty-nine transfer counter-current distribution in 70 per cent aqueous methanol with 80 per cent $CHCl_3$-20 per cent CCl_4 as the lower phase and was analyzed as described above. The radioactivity was separated from

the carrier estriol by this distribution, the peak for weight of estriol being in tube 25, and that for radioactivity in tube 0.

Redistribution of Estradiol—The contents of tubes 34 to 56 of the 99 transfer distribution (Fig. 2) were pooled. The pool contained 0.34 mg. of estradiol and 1150 c.p.m. (3380 c.p.m. per mg.). An additional 10.0 mg. of carrier estradiol were added, which brought the specific activity to 112 c.p.m. per mg. The estradiol was then subjected to a forty-nine transfer counter-current distribution in 70 per cent aqueous methanol with 40 per cent $CHCl_3$-60 per cent CCl_4 as the lower phase. The results of the analyses of this distribution are shown graphically in Fig. 3. Again, the distribution of radioactivity and weight of estradiol fit the same theoretical curve. Calculation of the area under the theoretical curve showed the

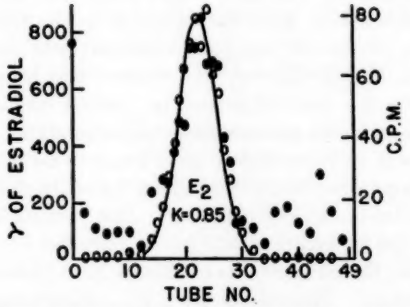

FIG. 3. Forty-nine transfer redistribution of estradiol. The upper layer is 70 per cent aqueous methanol, the lower layer, 40 per cent $CHCl_3$-60 per cent CCl_4. The upper layers were transferred. See Fig. 2 for an explanation of the symbols.

presence of 7.4 mg. and 695 c.p.m., thus giving a specific activity of 94 c.p.m. per mg. Calculation of specific activities for the individual tubes under the peak gave a value for the specific activity of 87 ± 8 (standard error of the mean, fifteen determinations).

Precipitation with Digitonin (20)—The contents of tubes 14 to 30 from the forty-nine transfer counter-current distribution (Fig. 3) of the estradiol were pooled. The estradiol was dissolved in 0.9 ml. of hot methanol with 30 mg. of digitonin. Hot water was added until the solution was faintly cloudy (0.3 ml.), and, after standing for an hour at room temperature, an additional 0.3 ml. of H_2O was added. While the solution was left to stand at 5° for 3 hours, a flocculent precipitate separated. The precipitate was centrifuged and washed twice with cold 60 per cent methanol, redissolved in 1.5 ml. of hot 60 per cent methanol, and again allowed to precipitate in the cold. This precipitate was centrifuged and washed with cold 60 per cent methanol. The mother liquors from the first and second precipita-

tions were kept separate. The residues from the two mother liquors and the precipitate were each dissolved in 1 ml. of anhydrous pyridine and heated on the steam bath for 1 hour. 10 ml. of absolute ether were added to each fraction, and the digitonin was removed by centrifugation. The ether solutions were washed twice with 1 ml. of H_2O and then evaporated to dryness *in vacuo*. Each fraction was counted and analyzed for estradiol by the fluorescence method. The first mother liquor contained 1.08 mg. of estradiol and 128 c.p.m. (119 c.p.m. per mg.). The second mother liquor contained 0.74 mg. of estradiol and 67 c.p.m. (91 c.p.m. per mg.); the final precipitate contained 2.98 mg. of estradiol and 276 c.p.m. (93 c.p.m. per mg.). In this purification procedure, therefore, there was no tendency for the radioactivity to separate from the estradiol.

Enzymatic Oxidation of Estradiol—Since there was no indication of a separation of radioactivity from the estradiol in the preceding step, the estradiol from the mother liquors and precipitate was pooled. The crystalline residue was slightly brown. To remove this brown color another phenolic separation was carried out. The neutral fraction contained no radioactivity; the estradiol was colorless and crystalline.

This estradiol was then incubated for 3 hours at 25° in the presence of oxidized diphosphopyridine nucleotide with a partially purified preparation of placental estradiol-17β dehydrogenase.[2] This enzyme has a high degree of specificity for the reversible oxidation of estradiol-17β to estrone.

After incubation, the mixture was extracted four times with 0.5 volume of methylene chloride. The residue from this extract was subjected to a phenolic separation. The phenolic fraction was then subjected to a twenty-four transfer counter-current distribution in 50 per cent methanol-carbon tetrachloride. A stainless steel machine with twenty-five tubes was utilized in this distribution (H. O. Post Scientific Instrument Company). The results of this distribution are shown in Fig. 4. Practically all of the estradiol ($K = 2.45$) has been oxidized to estrone ($K = 0.33$). The radioactivity was associated with the estrone. A single theoretical curve fits the data for radioactivity and weight of estrone. The distribution showed the presence of 2.28 mg. of estrone with 228 c.p.m. (100 c.p.m. per mg.). Calculation of specific activities of individual tubes under the peak gave a value of 101 $\pm$ 5 (standard error of the mean, nine determinations) c.p.m. per mg. The estrone from this distribution was pooled and recrystallized from aqueous ethanol. The recrystallized estrone weighed 1.14 mg. and contained 106 c.p.m. (93 c.p.m. per mg.).

Summary of Specific Activities—Table I shows the weight of estradiol

[2] We wish to express our sincere appreciation to Miss Lorna Langer of the Huntington Laboratories for furnishing this enzyme preparation and for information on its properties and use.

determined by fluorescence in H_2SO_4, the counts per minute in the estradiol, and the resulting specific activity at each step in the purification of the estradiol. It is apparent that the specific activity remained essentially constant throughout all purification steps subsequent to the 99 transfer counter-current distribution.

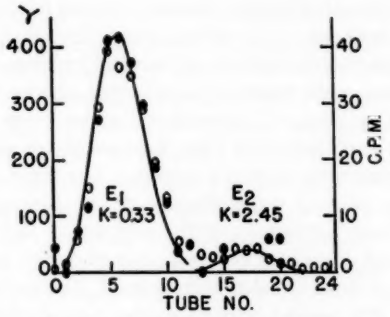

FIG. 4. Twenty-four transfer counter-current distribution of oxidized estradiol. The upper layer is 50 per cent methanol, lower layer CCl_4. The upper layers were transferred. See Fig. 2 for an explanation of the symbols.

TABLE I

Specific Activities of Estradiol at Various Stages of Purification

Step in purification	Weight, mg.	C.p.m.	C.p.m. per mg.
99 transfer counter-current distribution....	0.34	1150	3380
99 " " " + 10 mg. added carrier.....	10.3	1150	112
49 transfer counter-current distribution....	7.4	695	94
Regenerated from digitonide.....	2.98	276	93
Oxidized to estrone.....	2.28	228	100
Recrystallized estrone.....	1.14	106	93

These results, therefore, strongly indicate the radiochemical purity of the estradiol. A conversion of 1.2 per cent of the testosterone had occurred as calculated by isotopic dilution.

Neutral Compounds—The neutral fraction from this incubation of testosterone-3-C^{14} with ovarian tissue was fractionated by paper chromatography (21). Approximately 70 per cent of the activity was present as unchanged testosterone. Most of the remaining radioactivity was present in a compound whose mobility on paper chromatograms was consistent with Δ^4-androstene-3,17-dione. A small fraction of the radioactivity could not be moved from the starting lines.

DISCUSSION

The methods employed for the isolation and radiochemical purification of the estradiol have been selected as those most likely to remove possible labeled contaminants. The eight transfer distribution between alkali and toluene represents a systematic method for carrying out extraction procedures so as to determine whether a separation has taken place or whether the same material is present in both fractions. In this particular case, the original phenolic fraction contained only a small fraction of the total radioactivity. There was some doubt as to whether any of this radioactivity was really present in phenolic compounds, since some neutral material would certainly be partitioned into the aqueous phase and might account for all of the radioactivity in that fraction. The eight transfer counter-current distribution showed a definite separation into two fractions, thus indicating that a large percentage of the activity in the phenolic fraction differed from the bulk of neutral material. In an earlier experiment similar to the one described here, labeled testosterone was incubated with slices of stallion testis.[3] The eight transfer counter-current distribution was not used, and the estrone separated from the phenolic fraction contained radioactivity. After more extensive purification, the radioactivity was separated and shown to reside in testosterone present as a contaminant. An eight transfer distribution of the type described here would have eliminated this more tedious purification.

Counter-current distribution seems to be a particularly valuable method for studies of radiochemical purity; it offers a precise method for the determination of a partition coefficient with the opportunity for individual determinations which act as repeated analytical checks, one for each tube analyzed. If the radioactivity and weight of the carrier both have peaks in the same tube of a distribution, both must have the same partition coefficient within very narrow limits. Interaction between the carrier and a labeled contaminant is not likely since very dilute solutions are used in distributions. In order to detect a lack of radiochemical purity, a marked separation of carrier from a labeled contaminant is not required. If peaks in weight of carrier and in radioactivity are separated only by a single tube, this inhomogeneity is often apparent since different theoretical curves will be required to fit the two sets of data. The specific activity will vary from tube to tube in a systematic manner. Of course, the greater the number of distributions in different solvent systems carried out with failure to demonstrate a lack of homogeneity, the greater is the degree of certainty that the radioactivity resides in the carrier.

[3] Unpublished data.

The precipitation of estradiol as the digitonide without a change in specific activity is good evidence that the label is present in the estradiol. It would be expected that a radioactive impurity in low concentration would not be precipitated in appreciable amounts, even though in higher concentrations it might form the insoluble complex.

The oxidation of the labeled estradiol to estrone represents a chemical conversion employed to supplement the physical criteria for radiochemical purity. This conversion was carried out enzymatically so as to offer additional specificity to the reaction.

It would seem quite unlikely that a labeled impurity could accompany the carrier estradiol through such rigorous purification steps. The C^{14} almost certainly resides in the estradiol molecule.

Since the labeled carbon atom has not been located in the estradiol, the possibility of breakdown of the testosterone to small fragments, followed by synthesis of estradiol from the labeled fragments, must be considered. The recovery of most of the radioactivity in unchanged testosterone eliminates extensive breakdown. If breakdown did occur to some extent, considerable dilution of the fragments would be expected from other metabolic processes. It seems unlikely that as large a fraction of the total radioactivity as 1.2 per cent could find its way into estradiol by this pathway.

This conversion is of particular interest, since it involves profound changes in the molecule, loss of the angular methyl group, and aromatization. The mechanism for such a conversion is worthy of further study. The hydroxylation of carbon 19 may serve as the initial reaction which leads to the removal of this carbon atom. Meyer has recently demonstrated the conversion of Δ^4-androsten-19-ol-3,17-dione to estrone by endocrine tissue (22). The formation of Δ^4-androsten-19-ol-3,17-dione from androstenedione has been demonstrated in adrenal tissue (23). The introduction of a second double bond may also be an intermediate reaction. The loss of formaldehyde and the aromatization of a steroid containing a 19-hydroxy-3-keto-1,4-diene grouping has been demonstrated under mildly alkaline conditions (24).

The rôle of the conversion of testosterone to estradiol in normal biosynthesis of the estrogens is not certain. This conversion could well account for the increased excretion of estrogens following the administration of testosterone; however, this increased excretion has been observed in castrated humans (9, 11) and in an adrenalectomized, castrated human (25). Hence, the rôle of endocrine tissue in the conversion may be questioned. However, in another incubation study carried out in a manner similar to the one presented here, ovaries removed from a woman early in a menstrual cycle failed to carry out the conversion. In other experi-

ments, the conversion of testosterone to estradiol has been demonstrated to be carried out by stallion testes, a known source of estrogenic steroids, and by a feminizing adrenal tumor. In addition to labeled estradiol, labeled estrone was also formed by the tumor.[3] These few correlations of the conversion with known sources of estrogen biosynthesis are suggestive of the importance of the conversion in the normal biosynthetic pathway.

SUMMARY

C^{14}-labeled estradiol-17β has been isolated following the incubation of testosterone-3-C^{14} with human ovarian slices. Δ^4-Androstene-3,17-dione was the only other compound identified as a conversion product.

The authors wish to express their sincere appreciation to Dr. Howard Ulfelder and Dr. Joe V. Meigs for furnishing the human tissue used in this study. They also wish to thank Dr. Marcel Gut for the preparation of the labeled testosterone, prepared from $BaC^{14}O_3$ obtained on allocation from the United States Atomic Energy Commission. For a generous gift of Equinex and A. P. L., the authors are grateful to Ayerst, McKenna and Harrison, Ltd.

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ISOLATION OF MELANOCYTE-STIMULATING HORMONE FROM HOG PITUITARY GLAND*

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The present communication is concerned with two substances possessing melanocyte-stimulating hormone (MSH) activity, α-MSH and β-MSH, found in hog pituitary gland. The main component, α-MSH, was isolated by a four-step process consisting of solvent extraction and fractional precipitation, oxycellulose adsorption, counter-current distribution, and paper electrophoresis. α-MSH has been found to be homogeneous with respect to counter-current distribution and to paper electrophoresis at different pH values. A preliminary report on the isolation of α-MSH appeared elsewhere (1). These hormones, which are produced in the intermediate lobe of the pituitary gland (2), previously were called the melanophore hormone, melanophore-expanding hormone, or intermedin. It has been known for several years that the skin of aquatic animals could be darkened by injections of these hormones (3). Recently, a similar effect was observed in human beings (4).

EXPERIMENTAL

Method of Assay—MSH was determined by an assay *in vitro* by using isolated skin from *Rana pipiens* frogs (5). The changes in skin color before and after addition of MSH were measured with a photoelectric reflection meter. Within limits the logarithm of MSH concentration was proportional to the ratio of the changes in reflectance obtained with an unknown and standard quantity of hormone. A unit of MSH is defined as the degree of darkening produced by 0.04 γ of a lyophilized water extract of beef posterior pituitary powder on isolated frog skin (5).

Starting Material—Early in the investigation it was recognized that acetone-dried posterior pituitary powder from hogs had approximately twice the MSH activity as that from cows. Although it was not known

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whether this was due to a different concentration of MSH or to a variation in chemical structure of the hormones from the two sources, it was decided to use the more potent material from hogs as the starting substance. Posterior pituitary powder had about 10 times the MSH activity as that from the anterior portion of the gland.

Preliminary studies of crude MSH preparations showed rapid dialysis of the active material through cellophane and collodion membranes. Hormonal activity was lost when MSH was mixed with undistilled solvents such as diethyl ether or butyl alcohol. The ninhydrin reaction resulted in complete loss of activity. Treatment of MSH with 0.1 N sodium hydroxide in a boiling water bath for 5 minutes modified its darkening properties. The alkali-treated material produced more prolonged darkening than the untreated preparation.

Isolation of MSH—The methods described below were used in the isolation of α-MSH.

The first step was a modification of the procedure devised by Payne *et al.* (6) for the extraction of corticotropin (ACTH). 100 gm. of hog posterior pituitary powder containing 3×10^7 MSH units per gm. were mixed with 20 ml. of acetone to give a smooth paste. 1 liter of glacial acetic acid was added and the mixture was heated on a hot-plate at 50° for 10 minutes with constant stirring. The supernatant fluid was removed after centrifugation, and the residue was extracted again with 1 liter of glacial acetic acid in the same manner except that the heating at 50° was omitted. The combined supernatant fluids (1900 ml.) were mixed with 0.5 volume of acetone (950 ml.) and then with 10 ml. of saturated sodium chloride solution. After remaining at room temperature for 15 to 20 minutes the mixture was centrifuged. The supernatant fluid (2700 ml.) was mixed with 2 volumes (5400 ml.) of cold petroleum ether and allowed to stand overnight at −10°. Two layers formed: a heavy precipitate settled as a hard cake at the bottom of the flask, and small amounts of precipitate collected at the interface. The fluid was decanted, and the precipitate adhering to the bottom of the flask was covered immediately with acetone. If addition of acetone were delayed, the precipitate formed a gummy mass which was difficult to manipulate. After several washings with acetone, a fine yellow powder resulted which could be dried readily *in vacuo*. Usually 16 to 18 gm. of product containing 1 to 2×10^8 MSH units per gm. were obtained. The yield with respect to MSH activity was approximately 85 per cent. This petroleum ether-precipitated fraction will be referred to as PEF.

8 gm. of PEF were dissolved in 160 ml. of 0.1 N acetic acid by gentle warming in a boiling water bath. An insoluble residue was removed by centrifugation at $18{,}000 \times g$. 16 gm. of washed oxycellulose (7) were

added to the supernatant fluid, and the mixture was shaken gently for 75 minutes and then centrifuged. The supernatant fluid was discarded. The oxycellulose was washed two or three times with 0.1 N acetic acid and then the active material was eluted by shaking the oxycellulose with 100 ml. of 80 per cent acetic acid for 60 minutes. After centrifugation the supernatant fluid was removed and centrifuged again at 18,000 $\times$ g to remove the last traces of oxycellulose. The separated portions of oxycellulose were saved for reelution in large batches to recover additional active material. The supernatant fluid was diluted with an equal volume of distilled water and lyophilized. The dilution step was necessary because solutions containing more than 40 but less than 100 per cent acetic acid could not be lyophilized with our equipment. Approximately 0.5 gm. of a pale yellow solid containing 1 to 1.5 $\times$ 10^9 MSH units per gm. was obtained. The MSH yield was approximately 50 per cent. This oxycellulose fraction was designated OXF.

In preparation for counter-current distribution, 2 gm. of OXF were dissolved in a mixture of 75 ml. of each phase of the solvent system, *sec*-butyl alcohol-0.5 per cent aqueous trichloroacetic acid, with the aid of a micro-Waring blendor at about $-10°$. The solvents were prepared by equilibrating equal volumes of 0.5 per cent aqueous trichloroacetic acid and redistilled *sec*-butyl alcohol at 5° for 24 hours. The resultant solution gradually separated into two layers and was used as the initial tube of a twelve tube distribution unit at 5°, with 75 ml. of each solvent phase being used per tube. The distribution proceeded with the lower phase advancing. MSH analysis of various fractions indicated that tubes 3 to 5 contained the major portion of MSH activity (Fig. 1). After completion of the run, contents of tubes 3 to 5 were combined and lyophilized. Traces of trichloroacetic acid left with the solid after lyophilization were removed immediately by repeated extraction with freshly distilled diethyl ether. At no time during this extraction was a given amount of ether allowed to remain in contact with the solid for more than a minute. The resultant light brown solid weighed 0.5 to 0.6 gm. and had an activity of 3 to 4 $\times$ 10^9 MSH units per gm. The MSH yield was approximately 75 per cent. This counter-current distribution fraction was designated CDF.

An electrophoretic apparatus with a 50 cm. horizontal migration path of the type described by Kunkel and Tiselius (8) was used to purify Fraction CDF further. The power supply had a capacity of 0 to 1000 volts and 0 to 50 ma. Whatman No. 3 MM filter paper strips, 11 $\times$ 58 cm., were used as the supporting medium. Fraction CDF was subjected to paper electrophoresis as follows: Pyridinium acetate buffer, pH 4.55, was prepared by mixing 2 volumes of 0.3 N acetic acid and 1 volume of 0.3 M aqueous pyridine solution. 40 mg. of CDF were dissolved in 0.6 ml. of

0.3 N acetic acid by warming slightly in a water bath. To the mixture was added 0.3 ml. of 0.3 M pyridine solution. The small amount of residual solid was removed by centrifugation of the solution at 18,000 × *g*. The supernatant fluid was applied dropwise to a filter paper strip. Great care was taken to confine the applied solution to a band no more than 1 cm.

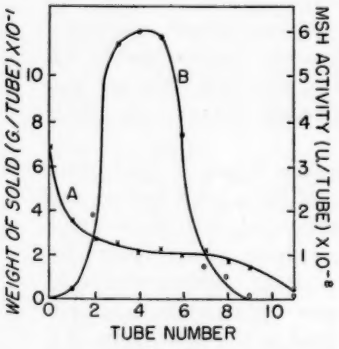

FIG. 1

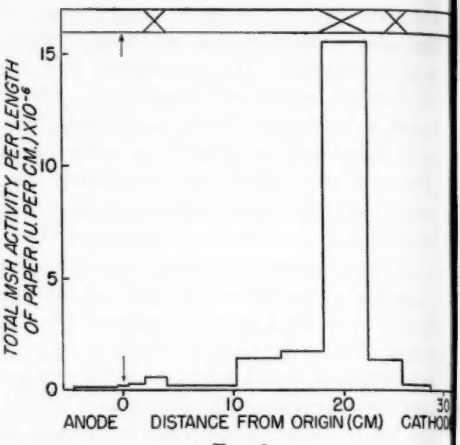

FIG. 2

FIG. 1. Twelve tube counter-current distribution of Fraction OXF in *sec*-butanol-0.5 per cent aqueous trichloroacetic acid system at 5°. Curve A represents weight of solid per tube. Curve B represents MSH activity of solid per tube. This preparation was obtained after solvent extraction and fractional precipitation of pituitary powder followed by oxycellulose adsorption and elution.

FIG. 2. Paper electrophoresis of Fraction CDF in pyridinium acetate buffer at pH 4.55, 18 volts per cm. of migration path, for 11 hours at 5°. The arrow indicates the site of application of the hormone. The crosses on the upper portion of the diagram indicate the location of prominent spots revealed by bromophenol blue staining. Fraction CDF was prepared by solvent extraction and fractional precipitation of pituitary powder, oxycellulose adsorption, and counter-current distribution in a twelve tube counter-current unit at 5°.

wide. The electrophoretic run usually lasted 10 to 12 hours at a potential of 18 to 20 volts per cm. of migration path and a current of 8 to 10 ma.

After completion of electrophoresis, the paper was frozen immediately to facilitate handling. A strip 1 cm. in width was cut along the whole length of the filter paper and dried for 5 to 10 minutes in an oven at 90°. After thorough drying the paper strip was made strongly alkaline by passage back and forth over a flask containing concentrated ammonium hydroxide. It was then stained for 30 seconds with 1 per cent bromophenol blue in 95 per cent alcohol saturated with mercuric chloride. The strip

was washed with a fine stream of 0.5 per cent acetic acid until all excess dye was removed. An electrophoretic pattern appeared as gray-blue bands which were converted to an intense blue color in the presence of ammonia vapor. The main MSH activity was associated with the second band located approximately 20 cm. from the origin in the direction of the cathode (Fig. 2).

The stained strip was used as a guide so that the active fraction could be cut from the remainder of the filter paper. MSH was eluted by shaking with 50 ml. of 3 N acetic acid for 60 minutes at 5°. The material was filtered

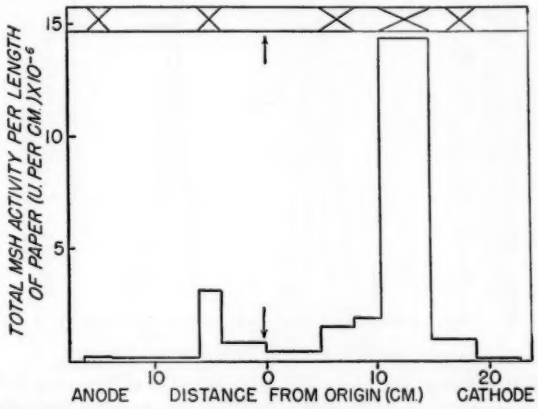

FIG. 3. Paper electrophoresis of Fraction CDF in sodium barbital buffer at pH 8.50, 18 volts per cm. of migration path, for 11 hours at 5°. Two MSH peaks were obtained. The arrow indicates the site of application of the hormone. The crosses on the upper portion of the diagram indicate the location of prominent spots revealed by bromophenol blue staining.

and lyophilized immediately. Approximately 4 mg. of a spongy white solid having an activity of 1 to 2 $\times$ 10^{10} MSH units per gm. were obtained. The MSH yield was about 40 per cent. This highly potent fraction was referred to as HPF-1.

An alternative procedure to the last step was tried in which Fraction CDF was submitted to two consecutive electrophoretic runs with an alkaline buffer in the first and an acidic buffer in the second. The electrophoretic runs were carried out as follows: 40 mg. of fraction CDF were dissolved in 1 ml. of sodium barbital buffer and submitted to electrophoresis in the same manner as previously described. Sodium barbital buffers prepared according to Michaelis (9) with pH values ranging from 8 to 9 were found effective. The active material was associated with the second band located approximately 12 cm. from the origin in the direction of the

cathode (Fig. 3) and was isolated as described above. After lyophilization the entire fraction was dissolved in 1 ml. of 0.2 N acetic acid for electrophoresis in the pyridinium acetate buffer at pH 4.55. Only a single band could be visualized in the stained guide strip. MSH analysis of different segments of the filter paper and ninhydrin tests on similar samples after acid hydrolysis showed single peaks which overlapped completely (Fig. 4). Extraction of the active region yielded about 1 mg. of a white spongy solid with an activity of 1 to 2 $\times$ 10^{10} MSH units per gm. The over-all yield

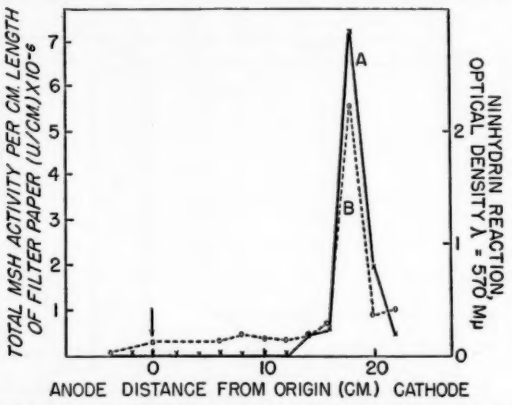

FIG. 4. MSH activity and ninhydrin color reaction of Fraction CDF after two paper electrophoretic runs. The active fraction was removed from the run at pH 8.0, 18 volts per cm. of migration path, for 8 hours and then tested at pH 4.55, 18 volts per cm., for 12 hours. Both runs were done at 5°. Curve A represents MSH activity. Curve B represents ninhydrin color reaction. The arrow indicates the site of application of the hormone.

of MSH in these two runs was approximately 10 per cent. This highly potent fraction was referred to as HPF-2.

Homogeneity of Purified MSH—The MSH preparations, HPF-1 and HPF-2, obtained after one and two electrophoretic runs, respectively, were tested for homogeneity by electrophoretic analysis with buffers ranging in pH from 1.40 to 12.2. The latter included glycine-sodium chloride-hydrochloric acid, pH 1.40, μ = 0.1; pyridinium acetate-acetic acid, pH 4.55, μ = 0.1; sodium barbital-sodium acetate-hydrochloric acid, pH 8.9, μ = 0.05; and glycine-sodium chloride-sodium hydroxide, pH 11.3, μ = 0.04; pH 11.9, μ = 0.1; and pH 12.2, μ = 0.1. The techniques used for electrophoresis were outlined previously. At all levels of pH tested, the hormone behaved as if it were a single component. In the electrophoretic run in which glycine buffer at pH 11.3 was used, MSH migrated only a

very short distance toward the cathode, being similar in this respect to dextran spots applied on both sides of the test material. Therefore, the isoelectric point of MSH was estimated to be within the range pH 10.5 to 11.0.

Homogeneity tests were carried out with a 100 tube, all glass counter-current distribution apparatus at room temperature (24 to 26°) with the upper phase advancing. The solvent system used was *sec*-butyl alcohol and 0.5 per cent aqueous trichloroacetic acid equilibrated at room temperature. At the end of a run the upper and lower phases were removed separately from each cell. The optical density at a wave-length of 2750 A

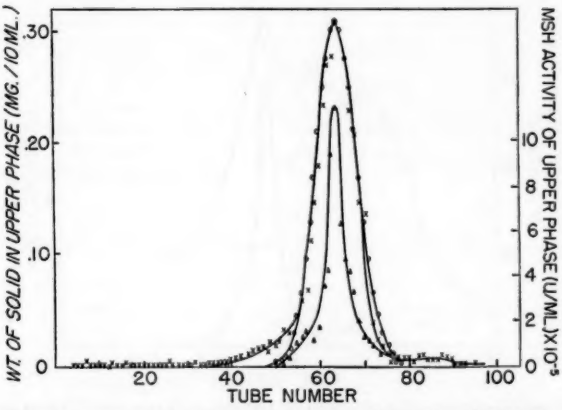

FIG. 5. Counter-current distribution of 20 mg. of Fraction HPF-1 in *sec*-butanol-0.5 per cent aqueous trichloroacetic acid system at room temperature; ×, experimental points based on weight of solid per 10 ml. of upper phase; ○, theoretical points; △, experimental points based on MSH activity per ml. of upper phase. This preparation was obtained from paper electrophoresis of Fraction CDF.

and the MSH activity were measured for each tube. The amount of solid in mg. per 10 ml. of upper phase was calculated from the optical density.

20 mg. of HPF-1 were distributed through 97 transfers during a 6 hour period. Comparison of the experimental distribution curve based on weight of solid per 10 ml. of upper phase with the theoretical curve calculated according to the usual procedure (10) revealed that approximately 95 per cent of the MSH was a single component (Fig. 5). The MSH activity curve was symmetrical and gave the same peak as the curve based on mg. of solid per 10 ml. of upper phase. From the position of the peak the distribution coefficient for MSH in the two solvents was calculated to be 2.1. Only about 50 per cent of the MSH used at the beginning of the experiment could be accounted for by the end of the run on the basis of MSH activity. When MSH was allowed to stand for 6 hours at room tempera-

ture in a test-tube containing the solvents used in the counter-current run, marked loss in activity occurred. Unfortunately, facilities were not available for carrying out the distribution at 5°.

The MSH activity curve was narrower than the theoretical curve or the curve based on weight of solid per 10 ml. of upper phase. This finding may have resulted from a greater loss of MSH activity on a percentage basis in the dilute tubes than in the concentrated tubes during the counter-current run. On the other hand, the rate of decrease in ultraviolet light absorption with time was the same in all tubes.

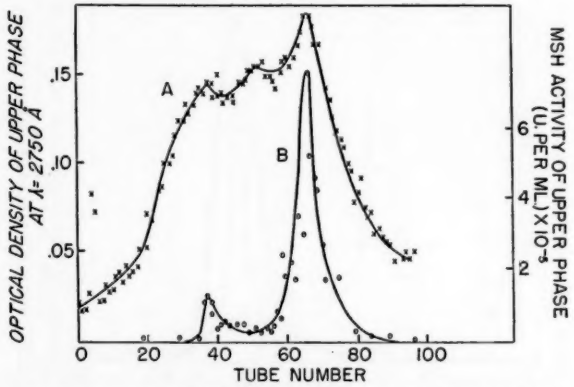

FIG. 6. Counter-current distribution of 100 mg. of Fraction CDF in *sec*-butanol-0.5 per cent aqueous trichloroacetic acid system at room temperature. Curve A represents optical density of upper phase at $\lambda = 2750$ A. Curve B represents MSH activity of upper phase. This preparation was obtained after counter-current distribution of Fraction OXF in a twelve tube counter-current unit at 5°.

Since the distribution curve was determined from the optical density and not on the basis of dry weight of solid per cell, it was possible that the preparation contained impurities which did not absorb light at the wavelength used. To clarify this point, samples from every fifth tube were subjected to acid hydrolysis for 10 to 12 hours and then allowed to react with ninhydrin. Only one ninhydrin-positive peak corresponding to the ultraviolet absorption and MSH activity peaks was found.

5 mg. of HPF-2 were examined by counter-current distribution in the same way through forty-nine transfers. Similar results were obtained. The calculated distribution coefficient of MSH in the two solvents was in exact agreement with the previous results.

Counter-Current Distribution and Moving Boundary Electrophoresis of Fraction CDF—When Fraction CDF was distributed through 97 transfers

in the large counter-current apparatus at room temperature, at least three peaks were found by ultraviolet absorption (Fig. 6). However, only one corresponded to a peak of great MSH activity. This occurred at the same position as the MSH peak of the purified hormone, Fractions HPF-1 and HPF-2 (Fig. 5), as well as that obtained with crude posterior pituitary powder (Fig. 8). A second relatively small peak also was obtained from Fraction CDF.

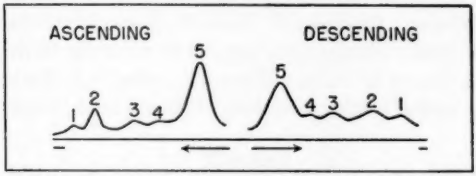

FIG. 7. Moving boundary electrophoresis of Fraction CDF in pyridinium acetate buffer, pH 4.55, 225 volts, 20 ma., for 173 minutes and 8 seconds at 2°. The arrows indicate the direction of migration.

TABLE I

MSH Activity of Fractions Obtained from Moving Boundary Electrophoresis of Preparation CDF

Fraction	Part of electrophoretic cell	Component (see Fig. 7)	Potency of solution	Potency of solid
			units per ml.	*units per gm.* $\times 10^9$
a_1	Ascending limb	1	0.15×10^6	0.2
a_2	" "	1 + 2	5.4×10^6	5.0
a_3	" "	1 + 2 + 3	7.2×10^6	3.0
a_4	" "	1 + 2 + 3 + 4	8.7×10^6	1.6
d_{3+4+5}	Descending "	3 + 4 + 5	6.7×10^6	0.3
Bottom	Bottom	All	8.5×10^6	Not determined
Original		"	2.3×10^7	4.0

In addition 100 mg. of Fraction CDF were studied by moving boundary electrophoresis in a standard electrophoretic cell at 2° with a pyridinium acetate buffer at pH 4.55, $\mu = 0.1$. Whereas only three peaks were found after counter-current distribution, electrophoresis showed five components with different mobilities moving toward the cathode (Fig. 7). A sampling device designed by Rigas *et al.* (11) was used to remove the solutions in the electrophoretic cell. Six separate fractions containing mixtures of the various components were obtained. These solutions were lyophilized and both the liquid and lyophilized powder were assayed for MSH activity. Results given in Table I show that the second peak possessed the main MSH activity and that this fraction corresponded to the active MSH obtained by paper electrophoresis.

Stability of MSH—It had been assumed that MSH was a relatively stable molecule because posterior pituitary powder could be kept at room temperature for an indefinite period without loss of activity. Furthermore, when mixtures of MSH were heated with dilute alkali, MSH activity was retained, whereas that of the other polypeptide hormones present was destroyed. Hence, we were surprised to find that highly purified MSH was very unstable. In order to determine the best conditions for storage, dry samples of the active hormone, HPF-1, were kept in sealed vials under a variety of conditions. As shown in Table II, it was found that MSH could be kept at −17° under nitrogen, oxygen, or *in vacuo* for 19 days with some, but not marked, loss of activity. However, under H_2S, the loss in potency during a similar period was appreciable. Storage for 50 days under air and

TABLE II

Stability of Purified MSH Preparation HPF-1

Sample No.	Atmosphere	Storage temperature	Time of storage	Potency after storage	Per cent of original potency
		°C.	*days*	*MSH units per gm.* × 10^6	
1	Vacuum	−17	19	7.7	59
2	Nitrogen	−17	19	7.0	54
3	Oxygen	−17	19	7.4	57
4	H_2S	−17	19	2.0	15
5	Oxygen	20–25	19	1.4	11
6	Vacuum	−17	50	2.5	16
7	Air	−17	50	3.5	22

in vacuo also caused a decrease in potency. The hormone was relatively unstable under oxygen at room temperature. Samples 6 and 7 were prepared in detergent-free vessels at the stage of electrophoresis, but glassware used at other times had been washed with detergents and rinsed well. No significant difference was observed.

Main MSH Component of Pituitary Gland—The following yields of MSH activity resulted from the four-step isolation process: solvent extraction and fractional precipitation, 85 per cent; oxycellulose adsorption, 50 per cent; counter-current distribution, 75 per cent; and paper electrophoresis, 10 to 40 per cent. Although the yield of MSH in each step was relatively high, the over-all yield was between 5 and 15 per cent. Thus, the question arose as to whether or not the hormone finally isolated represented the main MSH component of hog posterior pituitary gland. To help decide this point, posterior pituitary powder from hogs was distributed through 97 transfers in the counter-current distribution apparatus by using the same solvent system described previously. 1 gm. of hog pituitary powder was

shaken with a mixture of 20 ml. of each solvent phase at room temperature for 30 minutes. The insoluble residue was removed by centrifugation. 10 ml. of each phase of the extract were mixed and charged to the first tube of the counter-current distribution apparatus. The material was distributed with 10 ml. of each solvent phase through 97 transfers. The distribution curve based on MSH activity per tube as given in Fig. 8, Curve A, indicated the presence of two active components, which for simplicity were designated α-MSH and β-MSH. α-MSH (K = 2.1) and β-MSH (K =

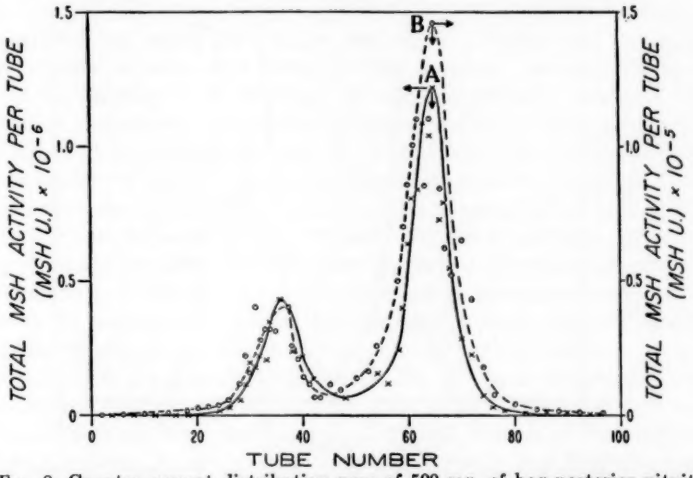

FIG. 8. Counter-current distribution runs of 500 mg. of hog posterior pituitary powder (Curve A, scale on left) and 500 mg. of hog anterior pituitary powder (Curve B, scale on right) in *sec*-butanol-0.5 per cent aqueous trichloroacetic acid system at room temperature. Two active components, α-MSH and β-MSH, were obtained in each case, but at about one-tenth the concentrations in the anterior powder.

0.6) represented about three-fourths and one-fourth, respectively, of the total MSH activity of the contents of all tubes. More than 70 per cent of the total activity present in the pituitary powder (0.5 gm.) was accounted for. These same components were observed in the distribution curves of the partially purified preparations, CDF and OXF, as indicated in Figs. 6 and 9, Curve B. Since the purified hormone preparation, HPF-1, and a partially purified fraction prepared according to the procedure of Landgrebe and Mitchell (12) each contained only one active component, α-MSH and β-MSH, respectively (Figs. 5 and 9, Curve A), it is unlikely that one of the active components was an artifact produced by the solvent system. Therefore, it can be assumed that two distinct melanocyte-stimulating

hormones are present in hog pituitary gland. When anterior pituitary powder from hogs was distributed in the same manner, α-MSH and β-MSH were observed in the same ratio and were characterized by the same distribution coefficient (Fig. 8, Curve B), as was the case with posterior pituitary powder, although there was a 10-fold difference in the total MSH activity. The striking resemblance between the two distribution curves suggests that these two melanocyte-stimulating hormones probably share a common site of synthesis in the pituitary gland, presumably the intermediate lobe.

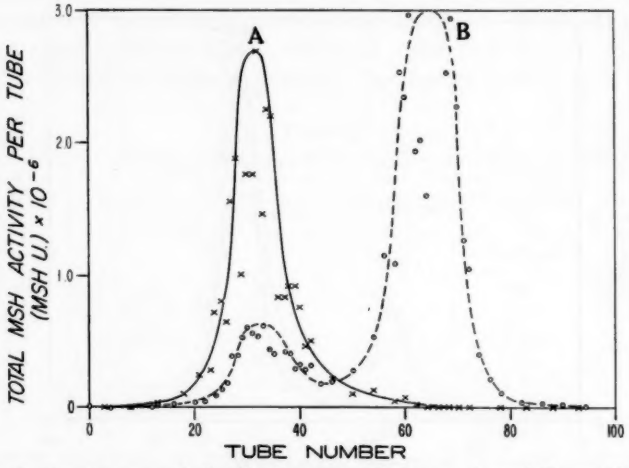

FIG. 9. Counter-current distribution runs of 16 mg. of partially purified MSH (Curve A) prepared according to Landgrebe and Mitchell (12) and 30 mg. of Fraction OXF (Curve B) in *sec*-butanol-0.5 per cent trichloroacetic acid at room temperature.

α-MSH has a specific potency of 1 to 2 $\times$ 10^{10} MSH units per gm. when purified by the procedure described previously. We have not determined the specific potency of β-MSH. It might be difficult to determine the relative potency of these two hormones because both are unstable and the measured potencies may not represent true values. The electrophoretic properties of inactivated MSH are identical with those of the active hormone. Thus any inactivated MSH present in preparations used as starting material for the final paper electrophoresis would appear in the end-product and cause undue lowering of specific potency. Hence, these two hormones must be prepared under identical conditions in order to compare their activities. Regardless of the specific activity of α-MSH, it is the main MSH component of hog pituitary gland, because the total α-MSH activity is greater than that of β-MSH.

In the past several reports have been made on purification of MSH from pituitary glands (12–16). In most cases the purified substance showed a moderate, but not marked, increase in potency over the starting material. However, recently Porath *et al.* (17) isolated a peptide which was homogeneous by ultracentrifugation and electrophoresis. It had about 1000 times the MSH activity of hog posterior pituitary powder, an isoelectric point at pH 5.2, and an approximate molecular weight of 3000. Geschwind and Li[1] reported isolation of a similar homogeneous preparation possessing a specific potency of 4 to 5 $\times$ 10^9 MSH units per gm., an isoelectric point at pH 5.5, and a distribution coefficient of 0.6 in the system *sec*-butanol-0.5 per cent trichloroacetic acid at 20°. Benfey and Purvis (18) also reported isolation of a highly purified fraction possessing 400 to 500 times the potency of the starting hog posterior pituitary powder. Their hormone preparation was similar in many respects to those prepared by Porath *et al.* and Geschwind and Li, except that it possessed a definitely basic isoelectric point.[2] These investigators apparently isolated β-MSH which accounts for less MSH activity in the pituitary gland than α-MSH.

In order to prove this point, a partially purified preparation, made from hog posterior pituitary powder according to the procedure described by Landgrebe and Mitchell (12),[3] was subjected to counter-current distribution in the same solvent system that was used in our homogeneity studies. The distribution curve (Fig. 9, Curve A), based on MSH activity per tube, indicated a single peak located at tube No. 32 which corresponded to a distribution coefficient of about 0.5. This value was close enough to be considered in the same range as that of β-MSH because of the magnitude of error that may be involved in estimation of distribution coefficients from the distribution curve based on MSH activity. When the same material was subjected to paper electrophoresis in sodium barbital buffer at pH 8.9, we observed one active component migrating toward the anode with a mobility very similar to that of β-MSH (Fig. 3) and several inactive components migrating toward both electrodes. No active component with the characteristics of α-MSH was observed in either case, whereas at a similar stage of purification our preparation of OXF definitely contained two active components as shown in Fig. 9, Curve B. Since Landgrebe and Mitchell used acetone precipitation at pH 6.5 in their fractionation procedure, it was suspected that a substance with a highly basic isoelectric point like that of α-MSH would remain in solution. As a matter of fact, it was found that the lyophilized mother liquor contained far more total

[1] Unpublished data.

[2] J. L. Purvis, private communication.

[3] This procedure was used by Porath *et al.* and also by Benfey and Purvis in their fractionation.

MSH activity than that of the active precipitate, although its specific potency was lower, owing to the dilution caused by the presence of large amounts of ammonium chloride. By means of paper electrophoresis, α-MSH was found and identified as the only active component in the lyophilized filtrate. Evidently, α-MSH was separated from β-MSH by acetone precipitation at pH 6.5 and was discarded in the filtrate during the

TABLE III

Molar Ratios of Amino Acids in Hydrolysates of α- and β-MSH

Amino acid	α-MSH	β-MSH*
Aspartic acid	1.2 (1)†	4.0 (4)†
Glutamic "	2.9 (3)	3.8 (4)
Serine‡	3.7 (4)	2.0 (2)
Glycine	3.1 (3)	4.0 (4)
Tyrosine	1.6 (2)	0 (Tentative)
Lysine	3.2 (3)	3.4 (3)
Arginine	2.0 (2)	2.2 (2)
Valine	2.3 (2)	0.4 (1)
Phenylalanine	2.9 (3)	2.0 (2)
Alanine	0.7 (1)	0.4 (1)
Cystine	0.4 (1)	0
Proline	3.0 (3)	5.0 (5)
Leucine	0.8 (1)	0.4 (1)
Threonine‡	0.6 (1)	0
Histidine	2.6 (3)	1.8 (2)
Methionine	2.0 (2)	1.4 (2)
Tryptophan§	2.0 (2)	Not determined
N-Terminal group	None (tentative)	Aspartic acid

Molar ratios were determined by the Levy procedure (19).

* The data were taken from the unpublished results of Geschwind and Li.

† The figures in parentheses represent suggested whole integers.

‡ The values for these two amino acids were not corrected for hydrolytic destruction.

§ Tryptophan was estimated from ultraviolet light absorption after subtracting tyrosine from the total value.

fractionation. This was probably the reason that the other investigators isolated β-MSH as the main active component. Therefore, the original fractionation procedure of Landgrebe and Mitchell was modified as follows: The 0.1 N hydrochloric acid eluate from oxycellulose was lyophilized directly instead of being precipitated with acetone. The resultant solid was subjected to paper electrophoresis in the same buffer mentioned before. One basic and one acidic active component, as well as several inactive components, were observed. The mobilities of the basic and the acidic active

components were very close to those of α-MSH and β-MSH, respectively (Fig. 3).

Amino Acid Composition of α-MSH and β-MSH—α-MSH (Fraction HPF-1) prepared by us and β-MSH isolated by Geschwind and Li were hydrolyzed and assayed for amino acids by the Levy procedure (19). Tryptophan was determined by ultraviolet absorption after subtracting tyrosine from the total value (20). Seventeen amino acids were found in α-MSH and their molecular ratios are presented in Table III. The analyses did not give quantitative recovery of amino acids. An estimation made from the alkali consumption during the treatment with 1,2,4-fluorodinitrobenzene accounted for only about 50 per cent of the sample tested. This low yield may be explained in part by the fact that tryptophan, anions, moisture, and ash were not included in the total amino acid percentage. In addition, it is possible that some impurities may have come from the filter paper used.

β-MSH contains thirteen known amino acids (Table III) and probably tyrosine and tryptophan. Aspartic acid was found to be the *N*-terminal group of β-MSH, whereas a similar sample of α-MSH yielded no *N*-terminal group when the 1,2,4-fluorodinitrobenzene method was used (21). It is evident from the data in Table III that α- and β-MSH have different amino acid compositions and that they are not the same as the corticotropins from hog and sheep (22–24). The molar ratios for histidine, aspartic acid, and glutamic acid are 3, 1, and 3, respectively, for α-MSH and 2, 4, and 4 for β-MSH. These findings are consistent with the fact that the isoelectric point of α-MSH is basic (pH 11) and that of β-MSH is acidic (pH 5.5).

DISCUSSION

The hormone obtained in the study reported here represents the main MSH component of hog posterior pituitary gland. The activity of the product was approximately 500 times greater on a weight basis than the starting material. However, purified MSH is unstable; it is possible that the potency of the hormone is greater than 1 to 2 $\times 10^{10}$ units per gm. as reported here. The purified hormone was homogeneous by paper electrophoresis at different pH values and by counter-current distribution. Nevertheless, other criteria of homogeneity remain to be satisfied.

The relation of ACTH to MSH has been controversial as discussed elsewhere (4). ACTH activity was not detected when MSH isolated in this study was tested by the method of ascorbic acid depletion. Also, vasopressin activity was not found. However, less than 0.5 mg. of MSH was used to test for ACTH and vasopressin; it is possible that larger quantities might show some biologic action, even though negligible, similar to those of ACTH and vasopressin. Assay in our laboratory of White's corticotropin

A (23) and Bell's β-corticotropin (24), which probably represent the main ACTH hormone of hog pituitary gland, showed 1.7×10^8 MSH units per gm. Thus, purified ACTH has 1 per cent MSH activity either on the basis of contamination or as an intrinsic part of the molecule. The molecular ratios of amino acids in ACTH and MSH are different (22–24). In addition, hog posterior pituitary powder has approximately 10 times as much MSH activity but only one-tenth as much ACTH activity as that from the anterior portion of the gland. According to the distribution of MSH and ACTH in the pituitary gland, the biologic properties of the purified hormones, their amino acid composition, and physical and chemical characteristics, they are distinct substances. MSH was not found to have any intrinsic ACTH activity, but ACTH could have as much as 1 per cent intrinsic MSH activity.

In spite of all the evidence indicating that the two hormones are separate, one remaining source of difficulty is that, in man and animals, elevated blood and urine levels of one hormone seem to be associated with an elevation in the other. Furthermore, when cortisone or hydrocortisone is given to a patient having increased levels of ACTH and MSH, both hormones are reduced to normal (4, 25, 26). It seems as if production of the two hormones is similar and is regulated by like factors.[4] This finding is not incompatible with individuality of the two hormones.

SUMMARY

It was demonstrated that two distinct melanocyte-stimulating hormones (α- and β-MSH) are present in hog pituitary gland. The main active component (α-MSH) was isolated from hog posterior pituitary powder as a fraction homogeneous by counter-current distribution and paper electrophoresis at different pH values. Physical and chemical properties and amino acid composition of α-MSH are described. MSH possesses no detectable ACTH or vasopressin activity.

We want to thank Dr. I. I. Geschwind and Mr. David Chung for carrying out the amino acid analysis of α-MSH and permitting us to use their results on the amino acid composition of β-MSH. Dr. Rigas assisted in the electrophoretic studies, and the Armour Laboratories and Wilson and Company, Inc., supplied us with a total of 3 kilos of hog posterior pituitary powder. Suggestions by Dr. H. S. Mason were appreciated. Mrs. Loleta Thompson gave valuable technical assistance.

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THE INHIBITION OF TRYPSIN

IV. REACTION WITH DIETHYL *p*-NITROPHENYL PHOSPHATE IN THE PRESENCE OF UREA*

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The inhibition of proteolytic enzymes by organophosphorus compounds has been extensively studied in recent years (1, 2). Such studies have shown that the reaction of trypsin with diisopropyl fluorophosphate (3) or diethyl *p*-nitrophenyl phosphate (DENP) (4) is accompanied by a loss in the proteolytic, esterase, and amidase activities of this enzyme. It is not certain, however, whether these inhibitors combine at the site responsible for the catalytic function of trypsin (4, 5).

In a previous paper in this series (6), it was shown that, although trypsin was inactivated by concentrations of urea ranging from 3 to 5 M, its enzymatic activity was retained in 6 to 8 M urea solution. Since most proteins are known to undergo rapid denaturation in high concentrations of urea, a more detailed examination has been made of the properties of trypsin in 8 M urea. During the course of such studies it was noted that, unlike native trypsin, the proteolytic activity of urea-treated trypsin was not inhibited by DENP. This observation prompted the present investigation, the object of which was to determine whether DENP reacts with trypsin at a site which differs from the site responsible for the catalytic function of the enzyme.

EXPERIMENTAL

Materials

The trypsin employed was a twice crystallized product containing 50 per cent $MgSO_4$ purchased from the Worthington Biochemical Corporation, Freehold, New Jersey. The enzyme was rendered free of salt by dialysis against 0.001 N HCl, followed by lyophilization. A fresh stock solution of trypsin was prepared prior to each experiment, and the concentration of trypsin protein (mg. per ml.) was determined spectrophotometrically at

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XUM

280 mμ by a conversion factor of 0.586 (7). The desired concentration of trypsin was obtained by suitable dilution of the stock solution.

DENP was kindly furnished by Dr. William Summerson of the Chemical Corps Medical Laboratories, Army Chemical Center, Maryland. A stock solution of 3×10^{-2} M, prepared in isopropanol and stored at 4°, was diluted to the desired concentration with isopropanol just prior to its use.

Diethyl phosphoryl trypsin (DEPT) was prepared by treating 90 mg. of trypsin in 5 ml. of 0.05 M borate buffer, pH 7.6, containing 0.01 M $CaCl_2$, with 2 ml. of the stock DENP solution for 48 hours at room temperature. After removal of the unchanged DENP by dialysis, proteolytic activity was measured on casein as described below. Treatment with successive 2 ml. portions of stock DENP solution was continued in this fashion until there was no further loss in activity. Three such treatments yielded a preparation which had from 0.5 to 1.0 per cent of the activity of a control enzyme solution which lacked DENP. DEPT was also prepared in the presence of urea by the same procedure with borate buffer containing 8.3 M urea. In this instance, however, precipitation of the protein occurred upon dialysis, which made it impossible to measure its activity. Three successive treatments with DENP were therefore assumed to yield completely changed DEPT. Both preparations of DEPT were lyophilized and stored in a dry state.

Methods

Proteolytic activity was determined by an adaptation of the method of Kunitz (8), either 2 per cent casein or 2 per cent denatured hemoglobin dissolved in 0.05 M borate buffer, pH 7.6, being used. Hemoglobin was denatured by using a buffer solution containing 6.7 M urea (9). To the enzyme solution in a volume of 1 ml. was added 1 ml. of the borate buffer, followed by the addition of 3 ml. of substrate solution. After a digestion period of 10 minutes at 37°, the reaction was stopped with 5 ml. of 5 per cent trichloroacetic acid, and the absorbance of the filtrate was read at 280 mμ.

Esterase activity was measured by the spectrophotometric method of Schwert and Takenaka (10) with α-*N*-benzoyl-L-arginine methyl ester as the substrate.

The effect of DENP on the proteolytic and esterase activities of trypsin in the presence or absence of urea was studied as follows. To 600 γ of trypsin in 4 ml. of 0.05 M borate buffer, pH 7.6, containing 8.3 M urea was added 1 ml. of 6×10^{-3} M DENP in isopropanol. A similar system from which urea had been omitted contained 0.02 M $CaCl_2$ to minimize self-digestion of the enzyme (11–13). 1 ml. of isopropanol replaced the DENP solution in controls set up to measure losses in activity in the absence of

DENP. Enzyme-free controls were also necessary to correct for the spontaneous hydrolysis of DENP during the course of the reaction. The reaction was allowed to proceed at 25°, during which time the liberation of *p*-nitrophenol (PNP) was measured according to the method of Hartley and Kilby (14), and the proteolytic (casein substrate) and esterase activities on 0.1 ml. aliquots were determined as described above. The reaction was assumed to be complete when no further increase in PNP could be detected (usually after 48 hours, although final readings were recorded at the end of 72 hours). The maximal PNP released under these

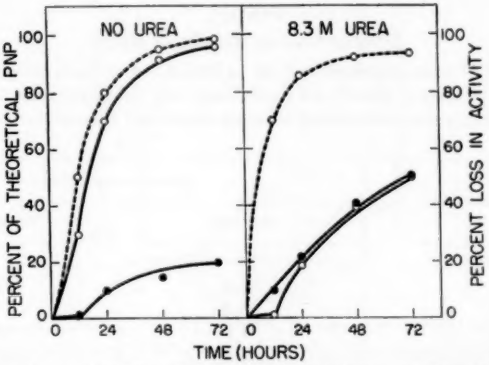

FIG. 1. The release of PNP and the loss in proteolytic activity accompanying the reaction of DENP with trypsin in the presence or absence of 8.3 M urea. See "Methods" for experimental details. The ordinate on the left is the PNP liberated as a percentage of the theoretical maximum (broken curves). The ordinate on the right is the loss in proteolytic activity (on casein substrate) as a percentage of the initial activity (solid curves). ○, with DENP; ●, without DENP.

conditions in the presence or absence of urea was found to be 0.90 and 0.92 mole per mole of trypsin, respectively, assuming the latter to have a molecular weight of 20,000 (15). This corresponds rather closely to the theoretical value of 1.0 based on a stoichiometric reaction between DEPT and trypsin in a molar ratio of 1:1 (4).

Results

Reaction of DENP with Trypsin in Presence or Absence of Urea

The liberation of PNP and the changes in proteolytic activity of trypsin treated with DENP in the presence and absence of urea are recorded in Fig. 1. In the absence of urea, the rate at which PNP was liberated closely paralleled the loss in proteolytic activity, an observation similar to that reported earlier by Kilby and Youatt (4). In the presence of urea, a simi-

lar rate of release of PNP was obtained, but of particular interest is the fact that the proteolytic activity of the enzyme was essentially unaffected by DENP. The loss in activity that did occur could be accounted for by the loss in activity which was apparent in the control to which no DENP had been added. Although not included in Fig. 1, the changes in esterase activity were quite similar to those presented for the proteolytic activity of the enzyme.

To rule out the possibility that trypsin was merely catalyzing the hydrolysis of DENP, samples of DEPT prepared in the presence and absence

TABLE I

Effect of Urea on Activity of DEPT

100 γ of DEPT* were dissolved in 1 ml. of 0.05 M borate buffer containing various concentrations of urea to give 0 to 8 M solutions and, where indicated, 0.01 M $CaCl_2$. Proteolytic activity was determined on urea-denatured hemoglobin as described in the text.

Urea concentration	Per cent reactivation†	
	No $CaCl_2$	0.01 M $CaCl_2$
moles per l.		
0	10.0	21.0
2	8.0	18.0
4	4.0	18.0
6	1.0	12.0
8	0	6.0

* Based on the extinction coefficient of DEPT at 280 mμ ($E^{1\%}_{1\,cm.}$ = 15.4), a value which is about 10 per cent less than that reported for trypsin (17).

† Per cent of the activity of an equivalent level of trypsin as measured in the absence of urea or $CaCl_2$.

of urea were analyzed for total phosphorus by the method of Sumner (16). DEPT prepared in buffer alone and in buffered urea solution was found to contain 0.150 and 0.129 per cent total P, respectively (moisture-free basis). The calculated P content of DEPT is 0.155 per cent. The release of PNP must therefore reflect a chemical reaction between trypsin and DENP both in the presence and in the absence of urea.

Reactivation of DEPT

The observation that trypsin in the presence of urea could react with a stoichiometric amount of DENP without losing its enzymatic activity suggested the possibility that urea might be capable of restoring the activity of DEPT which had been prepared in the absence of urea.

DEPT was dissolved in various concentrations of urea with and without

$CaCl_2$ and its activity subsequently measured on urea-denatured hemoglobin. As shown in Table I, the greatest degree of reactivation is obtained when DEPT is dissolved in urea-free solution containing Ca ions. Increasing the concentration of urea caused a progressive decrease in the restoration of activity that was obtained. These data were contrary to what had been anticipated, and will be further considered in the discussion of these results.

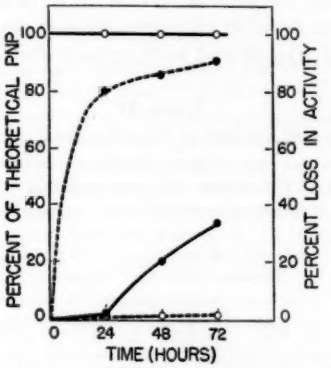

FIG. 2. The release of PNP and the loss in proteolytic activity accompanying the reaction of DENP with urea-treated trypsin in the presence and absence of cysteine. Each system contained 600 γ of trypsin in 4 ml. of 0.05 M borate buffer, pH 7.6, containing 8.3 M urea and 0.01 M $CaCl_2$, in the presence or absence of 0.005 M L-cysteine. After adding 1 ml. of 6×10^{-3} M DENP in isopropanol, the release of PNP and proteolytic activity were determined as described under "Methods." The ordinate on the left is the PNP released as a percentage of the theoretical maximum (broken curves). The ordinate on the right is the loss in proteolytic activity (on casein substrate) as a percentage of the initial activity (solid curves). ○, with cysteine; ●, without cysteine.

Influence of Cysteine

Although low concentrations of thiol compounds have been reported to have little, if any, effect on trypsin activity (18, 19), urea-treated trypsin was found to be rapidly inactivated by 0.005 M cysteine. As shown in Fig. 2, not only was urea-treated trypsin completely devoid of proteolytic activity in the presence of 0.005 M cysteine, but its ability to react with DENP was likewise destroyed. The reaction of urea-treated trypsin in the absence of cysteine is presented for comparative purposes. In this instance it is again evident that much of the proteolytic activity of the enzyme has been retained in spite of its ability to react with DENP, as indicated by the release of nearly the theoretical amount of PNP.

XUM

Evidence for Denaturation of Trypsin by Urea

The enhanced susceptibility of urea-treated trypsin to cysteine inactivation suggested the unmasking of disulfide linkages which often accompanies denaturation of proteins (20). Further evidence, relating to the possibility that urea-treated trypsin possesses the characteristics of denatured protein, was therefore sought.

Optical Rotation Studies—Advantage was taken of the fact that the optical rotation of many proteins is known to increase upon denaturation (21, 22). The optical rotation of trypsin exposed to 8.3 M urea was compared with untreated trypsin and with trypsin which had been denatured

TABLE II

Optical Rotation of Urea-Treated Trypsin

In each system was 1 per cent trypsin dissolved in 0.05 M borate buffer, pH 7.6, containing 0.02 M $CaCl_2$. The buffer also contained 8.3 M urea in the case of the urea-treated trypsin. Proteolytic activity was measured on casein substrate as described in the text.

Time*	Trypsin in buffer		Trypsin in urea		Heat-treated trypsin†	
	$[\alpha]_D^{25.5}$	Loss in activity‡	$[\alpha]_D^{25.5}$	Loss in activity‡	$[\alpha]_D^{25.5}$	Loss in activity‡
	degrees	*per cent*	*degrees*	*per cent*	*degrees*	*per cent*
30 min	−45.4	0	−124.8	0	−150.0	100
18 hrs	−47.0	3.0	−124.8	0	−150.0	100

* All solutions were allowed to stand at room temperature (25.5°) for the time indicated.

† Autoclaved at 15 pounds pressure for 15 minutes. The precipitated material was redissolved in buffer solution containing urea.

‡ Compared to the initial activity at zero time.

by heat. The data presented in Table II demonstrate that trypsin dissolved in urea remained fully active, yet had an optical rotation almost 3 times as great as trypsin prepared in buffer alone. The rotation of heat-denatured trypsin was only slightly greater than that of the urea-treated trypsin.

Digestion by Tetrahymena Proteinase—From concurrent studies in this laboratory (23, 24), a proteinase, isolated from *Tetrahymena pyriformis* W, was available which was capable of attacking denatured proteins but unable to split native proteins. The susceptibility of urea-treated trypsin to attack by a crystalline preparation of this proteinase (24) was therefore studied. As can be seen from Table III, native trypsin was not attacked by the proteinase, and the slight loss in activity which ensued was the same in the presence or absence of the proteinase. The urea-treated trypsin,

on the other hand, was readily digested under the same conditions as those revealed by the absorption of the split products at 280 mμ. Unfortunately, the presence of cysteine which is required to activate the *Tetrahymena* proteinase (24) caused a complete loss in the activity of trypsin, even in the urea system lacking the proteinase. This made it impossible to assess the effect of digestion *per se* on the activity of trypsin. It is interesting to note that the activity of trypsin in the absence of urea was not affected by the amount of cysteine used to activate the proteinase.

TABLE III

Digestion of Trypsin in Presence or Absence of Urea by Crystalline Tetrahymena Proteinase

1 ml. of a 1 per cent trypsin solution in 0.05 M borate buffer, pH 7.5, containing 0.02 M $CaCl_2$, with and without 8.3 M urea, was treated with 0.25 ml. of proteinase solution (6 γ of enzyme N) containing 0.05 M L-cysteine. Control tubes containing cysteine without added proteinase were likewise included. After standing for 1 hour at 37°, a 0.1 ml. aliquot was removed for the determination of proteolytic activity on casein, and 1 ml. was deproteinized with 5 ml. of 5 per cent trichloroacetic acid. The absorbance of the filtrate was read at 280 mμ.

System	Trypsin in buffer		Trypsin in 8.3 M urea	
	Loss in activity*	Absorbance at 280 mμ	Loss in activity*	Absorbance at 280 mμ
	per cent		*per cent*	
Without proteinase	8	0	100	0
With proteinase	8	0	100	0.180

* Compared to the activity of systems in which the enzyme-cysteine solution was replaced by borate buffer.

DISCUSSION

The loss in enzymatic activity which accompanies the reaction of native trypsin with DENP would suggest that the site at which DENP reacts is normally so close to the catalytic site that the introduction of the diethyl phosphoryl moiety interferes with the hydrolytic function of the enzyme. That the site of reaction with DENP is not identical with the catalytic site, however, is strongly suggested by the observation that, in the presence of urea, the enzyme is capable of reacting with DENP without losing its ability to split peptide and ester bonds. The effect of urea may be visualized as an unfolding of the trypsin molecule due to the rupture of hydrogen bonds as postulated for other proteins (25). In so doing, these two sites may become spatially dissociated so that each becomes free to participate in its own peculiar function; *i.e.*, the liberation of PNP from DENP and the hydrolysis of peptide and ester linkages.

XUM

A further consequence of the structural change induced by urea appears to be the unmasking of disulfide linkages which are known to be present in trypsin (19). In the native molecule these disulfide linkages are not readily reduced (19), but, in the presence of urea, cysteine causes complete inactivation of trypsin with respect to its enzymatic activity as well as its reactivity towards DENP. It is suggested that the disulfide linkages provide restricting cross-linkages which protect the molecule from further unfolding in the presence of urea. Rupture of these linkages by reduction with cysteine may lead to a loss in activity in much the same manner as the inactivation of insulin by thiol compounds (26). Thus far, attempts to reactivate cysteine-inactivated trypsin have not proved successful; indeed, it is quite possible that the derangement of the molecule caused by the rupture of disulfide linkages may be an irreversible process (25).

Additional evidence that urea-treated trypsin represents an unfolded, expanded molecule characteristic of denatured protein is provided by (*a*) the marked increase in levorotation and (*b*) the enhanced susceptibility to digestion by the *Tetrahymena* proteinase which is known to attack denatured but not native proteins. While this work was in progress, Anfinsen *et al.* (27) reported that the enzymatic activity of ribonuclease was also retained under conditions in which the molecule exhibited the characteristics of denatured protein.

Although DEPT was essentially inactive when measured on casein substrate, 10 to 20 per cent of the original activity was obtained when urea-denatured hemoglobin was employed as the substrate. It seems likely that the urea contained in the hemoglobin substrate solution is responsible for this difference between the two substrates, the urea acting to unmask the catalytic site of the enzyme in accordance with the views expressed above. On the other hand, exposing DEPT to increasing concentrations of urea up to 8 M prior to the addition of the substrate reduced the extent of reactivation observed upon the addition of the urea-denatured hemoglobin. It may be that, in the absence of substrate, the unfolding of the DEPT molecule caused by urea has irreversibly exceeded the point required for catalytic activity. The presence of substrate may restrict the complete unfolding of the molecule so that its catalytic function is at least partially restored. This stabilizing effect of the substrate appears to be enhanced by Ca ions. The protective influence of substrate and Ca ions on the inactivation of trypsin by lower concentrations of urea, 3 M to 5 M, has already been pointed out (6).

SUMMARY

In the absence of urea, the reaction of diethyl *p*-nitrophenyl phosphate (DENP) with trypsin, as measured by the liberation of *p*-nitrophenol, was

accompanied by corresponding losses in proteolytic and esterase activities. In the presence of 8.3 M urea, the reaction between DENP and trypsin proceeded without concomitant losses in enzymatic activity.

Although ineffective against trypsin in the absence of urea, cysteine added to trypsin in the presence of urea completely destroyed its enzymatic activity as well as its reactivity towards DENP.

Although enzymatically inert towards casein, diethyl phosphoryl trypsin (DEPT) displayed 10 to 20 per cent of its original activity when urea-denatured hemoglobin was provided as the substrate. Exposure of DEPT to urea prior to the addition of the latter substrate tended to reduce the amount of reactivation that could be obtained.

An increased optical rotation and an enhanced susceptibility to digestion by the *Tetrahymena* proteinase support the view that urea-treated trypsin is in a denatured state.

Addendum—Subsequent to the acceptance of this paper for publication, several reports (28, 29) have appeared which indicate that trypsin treated with 8 M urea is actually enzymatically inactive and denatured but rapidly regains its activity when assayed by dilution under the conditions described under "Methods." The activity measurements recorded here reflect, therefore, the ability of the denatured enzyme to revert to its native, active form.

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ERYTHROCYTE METABOLISM

I. PURINE NUCLEOSIDE PHOSPHORYLASE*

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Previous studies from this laboratory on the preservation of erythrocytes stored *in vitro* have shown that the storage lesion, as characterized by the decline in level of organic phosphate esters, may be reversed by incubating the aged cells with certain purine nucleosides (1). Pyrimidine nucleosides were ineffective in this respect, as was the addition of the purine bases and ribose, alone or in combination. These observations, together with the fact that reincorporation of inorganic phosphate[1] into the organic phosphate fractions paralleled the restoration of viability (2), suggested that the initial reaction was being carried out by a purine nucleoside phosphorylase,

$$\text{Purine riboside} + P_i \rightarrow \text{purine} + \text{R-1-P} \qquad (1)$$

an enzyme whose existence in the erythrocyte had been suggested previously by the work of Dische (3), and more recently by Prankerd and Altman (4).

Preliminary reports of this investigation have established the rôle of nucleoside phosphorylase in the preservation of erythrocytes (5, 6). The present communication will describe the preparation, assay, and properties of this enzyme, which, in many respects, resembles the purine nucleoside phosphorylase found in liver (7) and brain (8).

EXPERIMENTAL

Materials—Whole blood, stored in ACD (National Institutes of Health, Formula B), was made available through the cooperation of Dr. Richard Czajkowski of the King County Central Blood Bank. Xanthine oxidase was isolated from cream by the method of Mackler *et al.* (9), through the first ammonium sulfate fractionation. Crystalline beef liver catalase was obtained from the Worthington Biochemical Corporation.

Calcium phosphate gel was prepared according to the method of Keilin and Hartree (10) and aged for at least 3 months before use.

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[1] The following abbreviations are used: P_i, inorganic phosphate; R-1-P, ribose-1-phosphate; R-5-P, ribose-5-phosphate; ACD, acid-citrate-dextrose medium; Tris, tris(hydroxymethyl)aminomethane.

Adenosine, inosine, and cytidine were obtained from the Schwarz Laboratories, Inc., uridine and thymidine from the California Foundation for Biochemical Research, xanthosine, xanthine, guanosine, hypoxanthine, and R-5-P from the Nutritional Biochemicals Corporation, uric acid from the Eastman Kodak Company, and ribose from the Mann Research Laboratories.

2,6-Diaminopurine riboside was generously supplied by Dr. G. B. Brown, adenosine mono- and trisulfate by Dr. A. Hock, and R-1-P by Dr. H. L. A. Tarr.

Methods—Since previous studies (7, 8, 11, 12) with nucleoside phosphorylases from various sources had shown that Reaction 1 is actually a reversible equilibrium favoring the synthetic direction, it was desirable in the

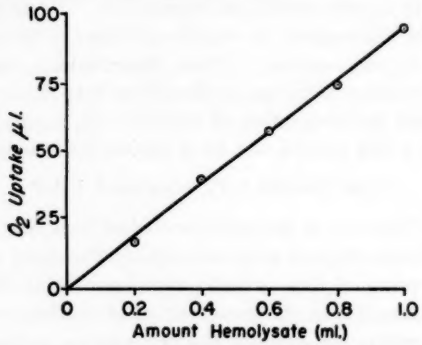

FIG. 1. Hemolysate prepared as described in the experimental section and subsequently diluted 1:10 for the experiment. Duration of experiment, 60 minutes.

present study to shift the equilibrium in the direction of breakdown. This has been accomplished either by oxidizing the liberated purine base, hypoxanthine, with xanthine oxidase (7) or by the use of arsenate to "trap" the pentose moiety.

For most purposes, the nucleoside phosphorylase may be assayed satisfactorily by coupling the system with xanthine oxidase and following the reaction manometrically. The manometer cups contained 10 μmoles of inosine, 100 μmoles of phosphate buffer, pH 7.5, 0.01 ml. (100 units) of catalase, 50 μmoles of ethanol, 0.1 ml. of xanthine oxidase (0.9 mg. of protein), and the phosphorylase, all contained in 2.8 ml. Inosine was omitted in the blanks. The center well contained 0.2 ml. of 6 M KOH; the cups were gassed with air and equilibrated in the water bath at 38° for 10 minutes before closing the stop-cocks. Over several hours, the rate of oxygen uptake was linear with time and linear with phosphorylase concentration (*cf.* Fig. 1), provided that the total uptake did not exceed 125 μl. per hour. 1 unit of phosphorylase is defined as that amount which causes the uptake

of 1 microatom of oxygen per hour. Specific activity is defined as units of enzyme per mg. of protein. Protein was estimated by the biuret method (13) with crystalline bovine serum albumin as the standard, and hemoglobin was determined spectrophotometrically at 540 mμ (14).

For preparations of the enzyme which were freed from hemoglobin, it was possible to follow the coupled reaction of phosphorylase with xanthine oxidase by the spectrophotometric method of Kalckar, *i.e.* by following the appearance of uric acid at 290 mμ (7). The assay system in 1 cm.

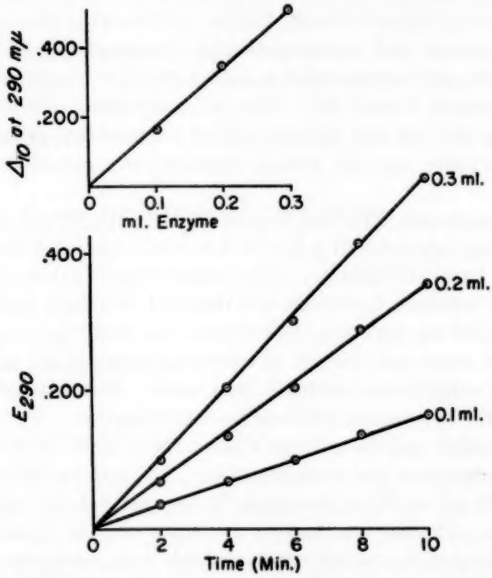

FIG. 2. Spectrophotometric assay as described in the experimental section

quartz cuvettes was identical to that above for the manometric assay, except that the phosphorylase was diluted so that the change in optical density at 290 mμ was less than 0.500 over a 10 minute period. Under these conditions, the activity was linear with time and proportional to enzyme concentration, as shown in Fig. 2.

The above assays require that inosine be used as substrate. A more general method, applicable to all reactive purine ribonucleosides, is obtained by substituting arsenate for phosphate and measuring the ribose liberated.

$$\text{Purine-ribose} + \text{arsenate} \rightarrow \text{purine} + \text{ribose-1-arsenate} \quad (2)$$

$$\text{Ribose-1-arsenate} + H_2O \rightarrow \text{ribose} + \text{arsenate} \quad (3)$$

The assay system contained 10 μmoles of the nucleoside, 20 μmoles of arsenate, 100 μmoles of Tris buffer, pH 7.5, phosphorylase, and water to make 3.0 ml. The tubes were sealed and incubated for 3 hours at 38°. At the end of this time, the tube contents were heat-deproteinized by immersion in a boiling water bath for 5 minutes, the denatured protein was removed by centrifugation, and 1.0 ml. samples of the supernatant fluid were taken for ribose analysis by using the method of Nelson for reducing sugars (15).

RESULTS AND DISCUSSION

Purification of Enzyme[2]—Fresh, human erythrocytes were washed three times by suspension and centrifugation in 3 volumes of cold 0.9 per cent sodium chloride and resuspended in saline solution to approximately the original hematocrit (about 50). The cell suspension was hemolyzed by quick freezing (dry ice and ethylene glycol monomethyl ether) and quick thawing three times, and the stroma fraction was removed by centrifugation.

90 ml. of hemolysate (Fraction I) were diluted with 360 ml. of cold water, the solution was taken to pH 5.4 with 1 N acetic acid, and the precipitate was removed by centrifugation. The supernatant fluid was adjusted to pH 7.5 with 1 N sodium hydroxide and dialyzed overnight against 10 liters of water. In 250 ml. portions, the solution was then readjusted to pH 5.5 and 600 ml. of water and 150 ml. of calcium phosphate gel (about 15 mg. per ml. of dry weight) were added in that order. The mixture was stirred mechanically for 15 minutes, followed by centrifugation. The supernatant fluid was discarded, and the gel was washed three times, each with 130 ml. of water by suspension and centrifugation. The enzyme was eluted from the gel with 60 ml. of 0.5 M phosphate buffer, pH 8.0, by suspension and centrifugation. The gel was treated as before with an additional 60 ml. portion of buffer and the two supernatant fluids were combined and dialyzed with mechanical stirring for 2 hours against water (Fraction II).

In 50 to 60 ml. portions, the dialyzed enzyme was brought as rapidly as possible to 60° and held at this temperature for 5 minutes. The solution was cooled rapidly in an ice bath, and the denatured protein was removed by centrifugation.

The supernatant fluid was brought to approximately 60 per cent of saturation by adding, in small portions, 42.6 gm. of solid ammonium sulfate per 100 ml. of solution. After the final addition, the solution was stirred gently for an additional 15 minutes, and the precipitate was recovered by centrifugation, dissolved in 8 to 10 ml. of water, and dialyzed for 2 hours

[2] Unless otherwise specified, all operations are carried out at 0–5° and centrifugations are performed at 2300 × *g* for 30 minutes in the International refrigerated centrifuge, model PR-1.

with stirring against water. After dialysis, any denatured material was removed by centrifugation. The supernatant fluid (Fraction III) may be stored in the frozen state or lyophilized and stored frozen as a dry powder. Under these conditions the enzyme remains active over several months of storage.

A summary of a representative purification procedure is given in Table I, where it is seen that there is about a 150-fold over-all purification from the hemolysate stage with a 50 per cent yield of enzyme recovered. In all preparations the specific activity of Fraction III varied between 300 and 500.

Since hemoglobin comprises more than 90 per cent of the total protein in hemolysates, it is evident that the removal of this chromoprotein constitutes the major problem in the purification of any erythrocyte enzyme. In the present procedure, the hemoglobin remains in solution and is dis-

TABLE I

Purification of Phosphorylase

Fraction No.	Volume	Phosphorylase activity	Protein	Specific activity
	ml.	*units*	*mg. per ml.*	*units per mg. protein*
I. Hemolysate	90	27,630	140*	2.2
II. Calcium phosphate gel eluate	125	22,700	2.7	67
III. 60% ammonium sulfate	12	13,800	3.4	340

* Based upon hemoglobin determination.

carded when the enzyme is adsorbed onto calcium phosphate gel. The phosphorylase, however, retains a reddish color when eluted from the gel, and this color is not diminished by repetition of the gel treatment. Even at the higher stage of purity (Fraction III), the enzyme has a reddish color. The absorption spectrum of the purified enzyme (peaks at 576, 408, 290 (shoulder), and 275 mμ) is somewhat similar to that of highly purified methemoglobin reductase (16), although no reductase activity can be demonstrated in the phosphorylase preparations.

Component Study of Reaction—By using the manometric assay system, wherein the phosphorylase reaction is coupled with xanthine oxidase, it was possible to demonstrate an absolute requirement for (1) the substrate, inosine, (2) phosphate (or arsenate), (3) the phosphorylase, and (4) xanthine oxidase (*cf.* Table II and Figs. 3 and 4). It was possible to show also a partial requirement for the catalase-ethanol system to remove hydrogen peroxide; the lack of an absolute requirement for the latter system indicated that one of the enzymes in the assay still contains traces of endogenous catalase.

In manometric experiments in which limiting amounts of substrates (1 to 2 μmoles) were used in order to obtain complete oxidation, it was observed that an additional 1 microatom of oxygen was taken up when inosine was compared with hypoxanthine. It would appear, therefore, that the ribose phosphate, formed in the phosphorylase reaction, is metab-

TABLE II

Component Study

Manometric assay as described in experimental section, except that the complete system contained 100 μmoles of Tris buffer, pH 7.5, and 20 μmoles of phosphate. Reaction time, 210 minutes.

Component omitted	O_2 uptake
	microatoms
None	34.3
Phosphorylase	0.5
Xanthine oxidase	0.1
Phosphate	5.1
Arsenate, replacing phosphate	25.0
Ethanol and catalase	27.8

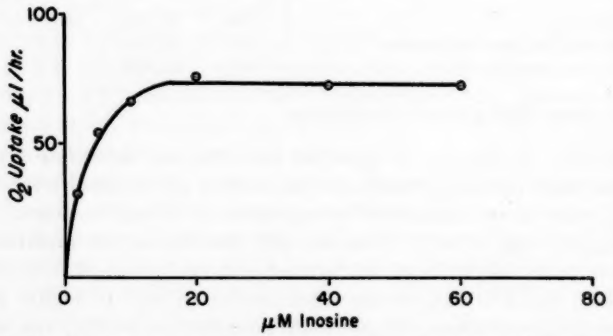

FIG. 3. Manometric assay as described in the experimental section with amounts of inosine as indicated.

olized further along an oxidative pathway. In this connection, it is of interest to recall the observation of Francoeur and Denstedt (17) that ribose-5-phosphate is peroxidized by H_2O_2 in the presence of catalase, although the oxidation product was not characterized. The further metabolism of the pentose moiety, liberated in erythrocyte phosphorylase reaction, is presently under investigation.

Products of Reaction—During the initial phases of this investigation it had been determined that the enzyme had an absolute requirement for

phosphate or arsenate (*cf.* Fig. 4), and that in the latter case the liberated ribose could be measured colorimetrically. When inosine was used as substrate, the reaction product, hypoxanthine, could be linked to xanthine oxidase. These observations, and the other close similarities between the erythrocyte enzyme and its counterparts in liver (7) or brain (8), left little doubt that the phosphorylase reaction in this case proceeded according to Reactions 1 to 3. Additional evidence for this assumption was obtained from experiments wherein aliquots from phosphorylase reaction mixtures were subjected to paper chromatography. The purine nucleosides may be

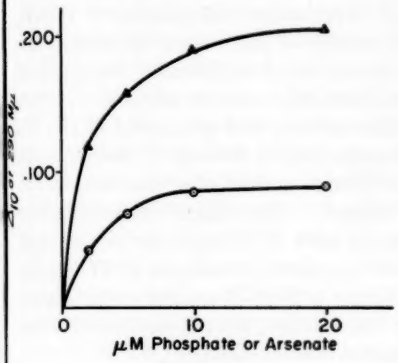

FIG. 4

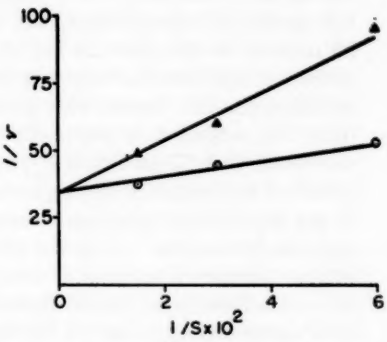

FIG. 5

FIG. 4. Spectrophotometric assay as described in the experimental section with the indicated amounts of phosphate (△) and arsenate (⊙).

FIG. 5. Spectrophotometric assay as described in the experimental section with the phosphate concentrations expressed in reciprocal units on the abscissa. Individual determinations in the absence (⊙) and presence (△) of 2 μmoles of pyrophosphate were made. Initial velocity (v) ($\Delta \log I_0/I$ at 290 mμ over a 10 minute interval) is expressed in reciprocal units on the ordinate.

separated from the free bases by chromatography in Solvent System A, isobutyric acid-water-concentrated ammonia (66:33:1), and located by inspection under ultraviolet light (Mineralite). Under these conditions, it was shown that hypoxanthine (R_F = 0.63) was released from inosine (R_F = 0.50) and guanine (R_F = 0.66) from guanosine (R_F = 0.53). In addition, the nature of the ribose moiety released was ascertained by chromatographing aliquots of reaction mixtures in Solvent System A and in a second system, Solvent System B, methanol-88 per cent formic acid-water (70:25:5), and by making use of the molybdate spray reagent (18) to locate phosphate esters and aniline trichloroacetate reagent (19) to locate free sugars. As expected, in the presence of arsenate, free ribose (R_F in Solvent System A = 0.48; R_F in Solvent System B = 0.67) was the only

reaction product. When phosphate was substituted for arsenate, R-1-P (R_F in Solvent System A = 0.24; R_F in Solvent System B = 0.54) was the principal product.[3] In crude hemolysates or preparations of the partially purified enzyme, several phosphate esters in addition to R-1-P were observed on the paper chromatograms. One of these has been identified by comparison with a known standard as R-5-P (R_F in Solvent System A = 0.23; R_F in Solvent System B = 0.67). This substance undoubtedly arises from R-1-P through the action of phosphoribomutase in erythrocytes (20).

Evidence that the erythrocyte phosphorylase can catalyze Reaction 1 in the direction of nucleoside synthesis can be obtained also by paper chromatographic methods. In a typical experiment, 100 μmoles of R-5-P, 100 μmoles of hypoxanthine, and 20 μmoles of Mg^{++} were incubated for 90 minutes at 37° with 20 ml. of stroma-free hemolysate. Neutralized perchloric acid filtrates were prepared from this reaction mixture. Hypoxanthine was determined with xanthine oxidase, and an aliquot of the filtrate was subjected to paper chromatography in Solvent System A. It was found that 77 μmoles of hypoxanthine remained after incubation (23 μmoles of hypoxanthine having been utilized). Quantitative densitometry[4] of the paper chromatograms indicated a ratio of 0.28 for the inosine and hypoxanthine areas. From the above enzymatic estimation of 77 μmoles of hypoxanthine remaining, it follows that 0.28 × 77 μmoles or 22 μmoles of inosine have been formed during the reaction, an amount which is in good agreement with that of the hypoxanthine utilized.

Kinetic Properties of Enzyme—The enzyme displays a broad pH optimum in the range pH 7.0 to 8.5. There is no diminution of activity when phosphate buffer is replaced by Tris or glycylglycine buffer, provided that phosphate or arsenate is present in catalytic amounts.

The effects of inosine, phosphate, and arsenate concentrations upon the reaction are illustrated in Figs. 3 and 4, respectively. The Michaelis constant (K_m) values, calculated by the Lineweaver-Burk method (21) from the double reciprocal plots, are 1.0×10^{-3} M for inosine, phosphate, or arsenate. The K_m value for inosine with the purified enzyme is of the same order of magnitude as noted previously for the over-all utilization of nucleosides during the rejuvenation of intact stored cells (1). It is also in good agreement with the value reported by Robins *et al.* (8) for the brain

[3] It is of interest that R-1-P gives a yellow color with the molybdate spray reagent (18) and a brown color with the aniline-trichloroacetate reagent (19), whereas glucose-1-phosphate, which moves with essentially the same R_F value in these solvent systems, gives a blue color with the first reagent and no reaction with the second. This difference in behavior is due undoubtedly to the greater lability of the phosphate ester linkage in R-1-P.

[4] Photovolt densitometer, model 301-A, phototube B, filter 5265, wave-length, 253 mμ.

enzyme, but considerably larger than that for the beef liver enzyme (11, 22). The K_m value for phosphate is likewise in good agreement with that reported for the brain enzyme (8), but again larger than that for the beef liver preparation (11). From Fig. 4 it can be seen that the maximal velocity obtained with arsenate is only 44 per cent of that with phosphate. This finding is in agreement with the value of 53 per cent reported by Rowen and Kornberg for the beef liver phosphorylase acting upon nicotinamide riboside (11).

Substrate Specificity—In a previous communication (6), it was pointed out that the crude hemolysate contains the necessary enzymes to split inosine, adenosine, guanosine, xanthosine, and 2,6-diaminopurine riboside. If the rate with inosine is expressed arbitrarily as 100, the relative rates of splitting of the other nucleosides are 65, 60, 40, and 25, respectively. The purified enzyme cleaves only inosine and guanosine with the relative rates of 100 and 60. Adenosine mono- and trisulfates, and the pyrimidine ribonucleosides, thymidine, uridine, and cytidine, are not cleaved in either the crude or purified systems. In addition, pyrimidine nucleosides do not inhibit the splitting of purine nucleosides. The above results are interpreted to mean that the purified enzyme splits only inosine and guanosine, in agreement with the substrate specificity noted by Kalckar for rat liver phosphorylase (7), and that in the crude hemolysate other enzymes must convert the other nucleosides to one of these two actual substrates. It is known that erythrocytes contain a powerful adenosine deaminase,[5] which is in keeping with our finding that equimolar amounts of ammonia are produced when aged erythrocytes are rejuvenated with adenosine (23). It is evident that adenosine is converted directly to inosine before phosphorolysis.

In preliminary experiments, it has been shown that ribose is liberated when xanthosine is split by crude hemolysates in the presence of arsenate. However, no oxygen uptake is observed when xanthosine is split by the phosphorylase-xanthine oxidase system. This would appear to rule out both the direct splitting of xanthosine to xanthine, or a conversion of xanthosine to inosine followed by splitting to hypoxanthine, since both hypoxanthine and xanthine would react in the xanthine oxidase system. Accordingly, the most plausible explanation would appear to be the conversion of xanthosine to guanosine, followed by phosphorolysis; this possibility is now being investigated.

Effect of Activators and Inhibitors—The enzyme is not inhibited by sodium Versenate or by fluoride, and this evidence, along with the lack of any demonstrable activation by a variety of metal ions, suggests that no metal ion is involved in the phosphorylase reaction. The enzyme is in-

[5] Bo Norberg, private communication.

hibited completely in the presence of 10^{-4} M *p*-chloromercuribenzoate or 10^{-3} M iodoacetate, which suggests that the enzyme contains essential thiol groups. An inhibition of 50 per cent in the presence of 10^{-4} M 2,4-dinitrophenol is of interest, since dinitrophenol is known to "uncouple" oxidative phosphorylation (24), a reaction similar to that of Reaction 1 wherein P_i is converted to organic phosphate. However, the inhibition of oxidative phosphorylation occurs at lower levels of dinitrophenol (10^{-5} to 10^{-6} M).

The enzyme is inhibited competitively by pyrophosphate, as shown by the plot of Fig. 5. From these data, a K_I value of 3.3×10^{-4} M may be calculated for pyrophosphate. The finding that pyrophosphate has a higher "affinity" for the enzyme than the natural cosubstrate, phosphate, is of interest, although the significance of this observation to erythrocyte metabolism is as yet unknown.

The authors are indebted to Miss Marion Hennessey, Mrs. Mary Jane Osborn, and Mrs. Cherie Howry for their expert assistance on this problem.

SUMMARY

1. Purine nucleoside phosphorylase has been purified about 150-fold from erythrocytes by means of adsorption and elution from calcium phosphate gel, selective heat denaturation, and ammonium sulfate fractionation.

2. The enzyme is specific for inosine and guanosine. In the presence of phosphate, the nucleoside is cleaved to the purine base and R-1-P. When phosphate is replaced by arsenate, ribose is the product.

3. Inosine, phosphate, and arsenate all have K_m values of 1.0×10^{-3} M with the erythrocyte phosphorylase.

4. The enzyme catalyzes both the breakdown and synthesis of inosine.

5. The phosphorylase is inhibited by *p*-chloromercuribenzoate, iodoacetate, 2,4-dinitrophenol, and pyrophosphate.

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STUDIES ON OXIDATION-REDUCTION

XXIV. OXIDATION-REDUCTION POTENTIALS OF FLAVIN ADENINE DINUCLEOTIDE*

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There have been several investigations of the oxidation-reduction potentials of riboflavin and alloxazine derivatives. Kuhn and Boulanger (1) reported the E_M potentials of numerous derivatives, including flavin mononucleotide (FMN). Michaelis, Schubert, and Smythe (2) and Michaelis and Schwarzenbach (3) thoroughly described the intermediate forms of oxidation-reduction of several flavins. The only reference to oxidation-reduction potentials of flavin adenine dinucleotide (FAD) is that of Ball (4), who reported $E_M = -0.250$ volt at pH 7.8 and 30°.[1] This dinucleotide is the most abundant flavin found in animal tissues (5). We report here a more complete potentiometric study of FAD between pH 2.4 and 12.4 and of FMN throughout the pH range 0.85 to 12.4. The results for FMN differ somewhat from those previously reported by Kuhn and Boulanger (1).

EXPERIMENTAL

The FAD used in the experiments reported here was prepared from Fleischmann's yeast and was isolated as the barium salt according to the procedure of Warburg and Christian (6). The dry weights of these samples were determined after drying small portions *in vacuo* over concentrated sulfuric acid at 40° for 12 hours. Different preparations varied consider-

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[1] E_M is the mid-point potential at a specified value of pH. Strictly, and as ordinarily defined, E_0 should be the mid-point potential when pH = 0. However, our data have not extended to low pH values except in the case of FMN, and in that case the number of points are too few to make a reliable estimate of pK_{O1}' (see foot-note 4). Accordingly E_0, calculated by extrapolation of data at relatively high pH values, must be considered a reference point and not a true value of E_0 as this quantity is defined.

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ably in the amounts of inorganic salts which they contained. The samples were analyzed for FAD, FMN, and riboflavin by the method of Burch *et al.* (7). 90 to 100 per cent of the total fluorescence was attributed to FAD. Paper chromatograms of the salt-free samples, prepared as described by Cramer (8), were examined for fluorescence under ultraviolet light. The preparations were free of riboflavin and contained only traces of FMN. The ratio of the optical density at 260 mμ to that at 450 mμ was between 3.70 and 3.80 (theoretical, 3.80). Flavin mononucleotide was kindly supplied by the Sigma Chemical Company. It should be emphasized that these compounds were free of apoenzyme.

The pH values were maintained constant by means of a series of 0.05 M buffers described by Clark (9). The pH values reported were determined with the hydrogen electrode. The calomel half cell potential was established with 0.05 M potassium hydrogen phthalate assumed to have the pH number 4.016 at 30° (10). To check the constancy of pH, solutions were tested with the glass electrode prior to and after titrations.

The titrations were performed at 30° in a kerosene bath. The apparatus was similar to that described by Clark (9) and Clark and Cohen (11), but constructed on a smaller scale to permit titrations of as little as 3 ml. of solution. The performance of the apparatus was checked by titrations of several oxidation-reduction systems of known potentials. Our results with riboflavin (Merck) agreed within 2 mv. of those reported by Michaelis *et al.* (2).

The samples were weighed on a micro balance and dissolved in appropriate buffer immediately before titration. The procedures were carried out in dim light, usually at night, to avoid decomposition of the light-sensitive material. Deaeration and mixing of the solutions were accomplished by bubbling with a stream of nitrogen passed over metallic copper at 500°. In titrations of FAD, slight drifts of potential were always in a positive direction. Below pH 9, the "drifts" at 50 per cent reduction were never greater than 1 mv. in 30 minutes. Equilibrium potentials were established on duplicate electrodes within a few minutes after addition of the reductant, and titrations could therefore be completed within 1 hour. At the higher concentrations employed in titrations of FMN, drifts in potential were negligible.

Reductive titrations were performed with dithionite and chromous acetate solutions. For titrations below pH 6, dithionite was tipped from a vial into 50 ml. of deaerated water containing 1 drop of 0.1 M NaOH. Above pH 6 the dithionite was added to deaerated buffer. Oxidative titrations were performed by addition of well aerated buffer to FAD solutions previously reduced with dithionite. Platinized asbestos and hydrogen never completely reduced FAD or FMN. Their use resulted in decompo-

sition, as evidenced by diminished optical density and fluorescence of the reoxygenated solution.

TABLE I

Reductive Titration of Flavin Adenine Dinucleotide

pH 4.88 (phthalate buffer); temperature, 30°; titrating agent, dithionite; flavin adenine dinucleotide concentration, 1.8×10^{-5} M.

Reducing agent	Equivalents of reducing agent	Semiquinone ($K = 0.09$)*	Reductant†	E_h (observed)	E_M† (calculated)	Deviation from average
ml.	*per mole FAD*	*per cent*	*per cent*	*volt*	*volt*	*volt*
0.03	0.069	3.60	1.65	−0.0534	−0.1071	−0.0001
0.05	0.115	5.10	3.25	0.0637	0.1072	−0.0002
0.07	0.161	6.25	4.93	0.0695	0.1069	+0.0001
0.09	0.207	7.25	6.32	0.0742	0.1074	−0.0003
0.14	0.322	9.05	11.59	0.0823	0.1074	−0.0003
0.19	0.437	10.40	16.64	0.0866	0.1059	+0.0011
0.24	0.552	11.40	21.89	0.0928	0.1073	−0.0003
0.29	0.667	11.50	27.58	0.0969	0.1072	−0.0002
0.34	0.782	12.60	32.78	0.1005	0.1071	−0.0001
0.39	0.897	12.90	38.38	0.1040	0.1071	−0.0001
0.44	1.011	13.00	44.93	0.1074	0.1071	−0.0001
0.49	1.126	12.90	49.87	0.1108	0.1070	0.0000
0.54	1.241	12.60	55.77	0.1144	0.1073	−0.0003
0.59	1.356	12.04	61.80	0.1180	0.1078	−0.0008
0.64	1.471	10.90	68.11	0.1219	0.1066	+0.0004
0.69	1.586	10.20	74.21	0.1267	0.1064	+0.0006
0.74	1.701	8.70	80.70	0.1325	0.1060	+0.0010
0.79	1.816	6.75	87.43	0.1412	0.1059	+0.0011
0.84	1.931	3.75	94.68	0.1621	0.1090	−0.0020
0.87	2.000	0	100.00			
Average					+0.1070	

* Calculated graphically by Elema's method (15, 16).

† Calculated by use of K as described by Michaelis (15, 16).

Results

It has been shown that in the oxidation-reduction process riboflavin forms a semiquinone (2, 3). In the case of FAD and FMN, the shape of the titration curves suggests the same sort of stepwise process. It is unlikely that the elongation of the curve is due to a mixture of two systems having slightly different E_M potentials. In several instances, fluorometric analysis of the solutions before and after titration showed that the FAD was not hydrolyzed appreciably at low pH or decomposed at high pH within the time required for titration. Comparison of oxidative and reductive titrations on the same sample of FAD showed that the effect of

the positive potential drifts, while insignificant in the range 5 to 95 per cent reduction, is to extend the end-points in the region of unpoised potentials in reductive titrations, while contracting the end-points in oxidative titrations. The extent of these effects is not easily evaluated or controlled. Consequently, in reductive titrations, it is difficult to ascertain how much of the elongation of the titration curve is due to residual oxygen and how

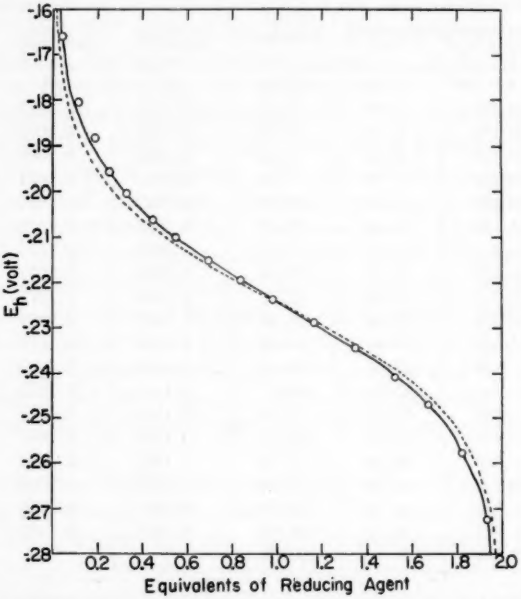

FIG. 1. Titration curve of FAD. Ordinate, E_h (hydrogen standard). Abscissa, equivalents of dithionite per mole of FAD; pH 7.14 (phosphate 0.05 M); FAD 10^{-5} M, 30°; unbroken curve, calculated for an "effective semiquinone formation constant," $K = 0.05$; broken curve, for a case of 2 equivalents without formation of semiquinone or dimer; $E_M = -0.2242$.

much is due to semiquinone formation.[2] For the purpose of description, and more particularly for calculating the mid-point by a first order approximation, that amount of reducing (or oxidizing) agent which was necessary to change the ratio of oxidant to reductant from 200:1 to 1:200 ($E_h = E_M \pm 0.07$ volt) was taken arbitrarily to represent 1.98 equivalents of reducing (or oxidizing) agent. Since the curves were symmetrical, the

[2] The method of Reed and Berkson (12), as applied to the rectification of titration curves by Clark and Perkins (13), is not applicable to cases in which titration curves are complicated by the formation of a semiquinone and dimers.

graphical mid-point was taken as the mid-point potential (E_M).[3] By the determination of end-points in this manner, the "effective formation constant," K, for semiquinone, was graphically evaluated by Elema's method (3, 15). With this constant, satisfactory calculations of E_M values for

TABLE II

E_M Values of Flavin Adenine Dinucleotide (30°)

$E_0 = 0.187$ (see foot-note 1). $E_{M7} = -0.219$ calculated.

pH	Buffer	Titrating agent	Concentration, FAD	E_M	Semiquinone
			M	*volt*	*per cent*
2.40	HCl	$CrAc_2$	7.5×10^{-5}	+0.0437	30
2.84	Phthalate	"	1.8×10^{-5}	+0.0188	33
3.99	"	"	1.5×10^{-5}	−0.0561	20
4.88	"	Dithionite	1.8×10^{-5}	−0.1067	13
6.20	Phosphate	"	6.7×10^{-6}	−0.1798	8
6.20	"	"	1.0×10^{-5}	−0.1838	10
6.28	"	$CrAc_2$	2.6×10^{-5}	−0.1848	6
6.55	"	Dithionite	1.0×10^{-5}	−0.2000	13
6.87	"	"	1.0×10^{-5}	−0.2102	13
6.90	"	"	3.9×10^{-7}	−0.2168	10
7.10	"	"	1.0×10^{-5}	−0.2236	15
7.14	"	"	1.0×10^{-5}	−0.2240	10
7.45	$Na_2P_2O_7$	"	3.9×10^{-7}	−0.2347	9
7.50	Borate	"	8.7×10^{-6}	−0.2348	10
7.55	"	"	1.0×10^{-5}	−0.2375	6
7.55	"	O_2	1.0×10^{-5}	−0.2367	10
8.50	"	Dithionite	6.5×10^{-6}	−0.2768	10
8.50	"	"	6.5×10^{-6}	−0.2760	11
8.65	"	"	7.5×10^{-5}	−0.2828	15
8.65	"	O_2	7.5×10^{-5}	−0.2810	10
8.90	"	$CrAc_2$	2.4×10^{-5}	−0.2850	12
10.30	Phosphate	Dithionite	1.6×10^{-5}	−0.3308	36
10.90	"	"	1.6×10^{-5}	−0.3648	20
11.76	"	"	1.7×10^{-4}	−0.4030	6
12.37	"	"	1.7×10^{-4}	−0.4290	0

[3] In previous studies of similar systems (2, 3), drifts of potential were not considered significant when pH was lower than 7. Our attempt to obviate this difficulty in part by our method of determining an equivalence point will yield somewhat lower estimates of semiquinone formation than the method used by Michaelis. He used asymptotes for reference. Experience in this laboratory strongly suggests that the more usual drifts of potential are due to residual oxygen in the nitrogen and adsorbed on the walls of vessels, and that this source of error becomes especially bothersome in titrations at high dilution and with reductants that are very sensitive to oxygen. Harbury (14) has given this matter special attention and has found some advantage in the use of pure helium for deaeration.

FAD were obtained by use of all the titrimetric data (Table I). The experimental points of a typical reductive titration of FAD with dithionite at pH 7.14 are shown in Fig. 1. The points correspond closely to the

TABLE III

E_M Values of Flavin Mononucleotide (30°)

E_0 = 0.187 (see foot-note 1, text).

pH	Buffer	Titrating agent	Concentration, FMN	E_M	Semiquinone or semiquinone dimer*
			M	*volt*	*per cent*
0.85	HCl	Dithionite	1×10^{-4}	+0.1395	44.5
2.22	Phthalate	"	1×10^{-4}	+0.0528	21.8
4.01	"	"	1×10^{-4}	−0.0542	10
4.05	"	"	1×10^{-3}	−0.0545	17.4
4.32	"	"	1×10^{-4}	−0.0725	11.1
4.70	"	"	1×10^{-4}	−0.0961	9.5
5.22	"	"	1×10^{-4}	−0.1261	10.6
5.62	"	"	1×10^{-4}	−0.1525	13.7
5.82	Phosphate	"	1×10^{-4}	−0.1603	12.9
6.22	"	"	1×10^{-2}	−0.2003	43
6.50	"	"	1×10^{-4}	−0.1933	11.5
7.00	"	"	1×10^{-4}	−0.2110	12.9
7.06	"	"	2×10^{-4}	−0.2172	15.4
7.09	"	"	4×10^{-4}	−0.2183	18.8
7.09	"	"	1×10^{-3}	−0.2185	24.0
7.14	"	"	1×10^{-4}	−0.2212	17.1
7.16	"	"	1×10^{-4}	−0.2202	18.8
7.74	Borate	"	1×10^{-4}	−0.2454	11.2
8.11	"	"	1×10^{-4}	−0.2560	17.6
8.16	"	"	1×10^{-4}	−0.2578	19.9
8.16	"	"	4×10^{-3}	−0.2585	18.0
8.16	"	"	1×10^{-3}	−0.2567	18.6
8.62	"	"	1×10^{-4}	−0.2739	17.1
8.90	"	"	1×10^{-4}	−0.2844	12.7
9.60	"	"	1×10^{-4}	−0.3086	9.1
10.49	"	"	1×10^{-4}	−0.3441	11.2
10.93	"	"	1×10^{-4}	−0.3671	6.8

* See the text.

theoretical values (line with open circle) calculated for a stepwise oxidation-reduction process having an "effective formation constant," K, equal to 0.05. The theoretical curve for a 2 electron system without semiquinone formation (broken line) is shown for comparison. In the titrations of FMN, end-points calculated in this manner coincided closely with the experimentally observed end-points (±2 per cent).

The oxidation-reduction potentials for the FAD system throughout the pH range 2.4 to 12.4 and for the FMN system throughout the pH range 0.8 to 10.9 are given in Tables II and III, respectively. The E_M values were obtained by determination of the point of symmetry of the respective titration curves. The amount of semiquinone present at the mid-point potential was calculated by methods previously cited (3). The E_M potentials of FAD and FMN throughout the pH range investigated are shown graphically by the E_M, pH, curve in Fig. 2.

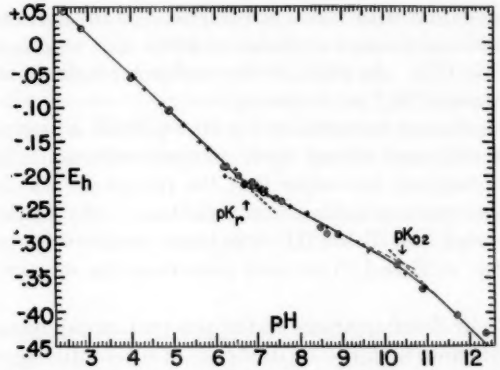

FIG. 2. Relation of E_M to pH at 50 per cent reduction, 30°. ⊙, FAD system; ×, FMN system; unbroken curve is for the FAD system. See the text for this equation, which applies only to a description of the data within the range of pH shown.

DISCUSSION

Dimerization of 1 molecule of oxidant with 1 molecule of reductant results in a steepening of the titration curve similar to that produced by semiquinone formation. In the case of the flavins, the two effects may be superposed (3). Resolution is achieved by a series of titrations at constant pH and successively increasing concentrations of flavin. The limiting titration curve for infinite dilution theoretically represents a system in which only oxidant, reductant, and semiquinone are formed. Further increase of the slope of the curves at higher concentrations is attributed to the formation of a dimeric compound. The per cents of semiquinone formed at 50 per cent reduction (Tables II and III) were calculated from the displacement of the experimental titration curve from the curve for a 2 electron oxidation-reduction process without semiquinone formation. Consequently they represent semiquinone and dimer formation, except in those instances, at constant pH, when dimerization was ruled out by data at several concentrations of flavin. On this basis there is no evidence for

the formation of a dimer of FAD at the concentrations employed. The per cents of semiquinone recorded in Table II, therefore, represent the amount of free radical formed under the specified conditions.

The calculations for per cent of semiquinone formed at 50 per cent reduction of FAD are subject to criticism because of the arbitrary method used in determining the equivalence points of titrations. Use of these values, however, brings the experimental points between 2 and 98 per cent reduction into close agreement with the theoretical values for an oxidation-reduction process with semiquinone formation (Fig. 1).

The determinations with FMN at pH 7.10 (± 0.10) indicate the formation of a significant amount of dimer of FMN and reductant in 10^{-3} M solutions (Table III). At pH 8.16 the tendency for dimerization at this same concentration (10^{-3} M) is absent.

At pH 6.22 and a concentration of 1×10^{-2} M FMN, a precipitate formed during the titration and cleared upon complete reduction. The titration curve was symmetrical, indicating that the precipitate was an aggregate (dimer) of equal parts of oxidant and reductant. The 43 per cent semiquinone calculated (see Table III) was based on the value of E_i on the assumption that at 25 and 75 per cent reductions the solution was homogeneous.

At pH <8 our determinations of the per cent semiquinone formed at 50 per cent reduction in dilute solutions are in substantial agreement with those determined for riboflavin (3). Above pH 8, however, the marked increase in per cent semiquinone formation reported for riboflavin was not observed for FAD and FMN. In our experience the titrations of FMN yield the most reliable data, since stable potentials can be obtained at concentrations where solubility is not a problem.

The experimental points of the E_M, pH, curve (Fig. 2) agree fairly well with the following theoretical equation in which E_M is the value of E_h at each mid-point.

$$E_M = E_0 - 0.0601 \text{ pH} + 0.03005 \log \frac{K_r' + (H^+)}{K_{O2}' + (H^+)}$$

This is derived on the assumption that the theoretical process is:

$$\text{Oxidant}^+ + 2e = \text{reductant}^-$$

Values of K_{O2}' and K_r' were determined by the intercepts obtained by extrapolation of the best straight lines drawn through the experimental points; $K_{O2}' = 4 \times 10^{-11}$, $K_r' = 2 \times 10^{-7}$. $E_0 = 0.187$ volt was obtained by extrapolating the experimental points to pH zero.[4]

[4] pK_{O2}' is used because of evidence in studies of other isoalloxazines of an ionization at very low pH. In the case of FMN the point at pH 0.85 (see Table III) deviates

SUMMARY

Potentiometric titration curves for flavin adenine dinucleotide (FAD) in the pH range 2.4 to 12.4 and for flavin mononucleotide (FMN) in the pH range 0.89 to 10.9 indicate that the oxidation-reduction process is reversible and involves the formation of a semiquinone as an intermediate in the 2 equivalent change. When appropriate corrections are made for positive drifts of potential encountered near the end-points in rapid titrations at low concentrations of FAD, the experimental points agree closely with the theoretical values calculated for a 2 equivalent change with semiquinone formation. The greater stability of potentials during titrations of more concentrated solutions of FMN increases the significance of the calculations for the amount of semiquinone formed at 50 per cent reduction.

At constant pH, the slopes of the titration curves for FMN increase with increasing concentrations of FMN and indicate the formation of a dimer.

For descriptive purposes the potentials at 50 per cent reduction (E_M) as a function of hydrogen ion activity (H^+) may be formulated by the equation:

$$E_M = E_0 - 0.0601 \text{ pH} + 0.03005 \log \frac{K_r' + (H^+)}{K_{O2}' + (H^+)}$$

where $E_0 = 0.187$ volt, $K_r' = 2 \times 10^{-7}$, $K_{O2}' = 4 \times 10^{-11}$.

The precision of the data is not such that distinctions can be made between values for FAD and values for FMN; no great difference would be expected. E_M at pH 7.0 $= -0.219$ calculated.

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from the curve of "0.06 slope." Unfortunately the number of points are inadequate for a reliable estimation of pK_{O1}' or for complete assurance that there is an inflection. For this reason Fig. 2 is restricted in the range of pH, and the equation given is to be regarded as descriptive only of the experimental data within the range of pH shown in Fig. 2. See also footnote 1.

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TRANSESTERIFICATION REACTIONS CATALYZED BY CHYMOTRYPSIN*

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As recently reported, the behavior of acetyl chymotrypsin with ethanol consists in a rapid transfer of the single acetyl group to that alcohol with the formation of ethyl acetate (1). Thus, the splitting of nitrophenyl acetates by chymotrypsin, faster in ethanolic than in aqueous solutions, may be explained by the formation of acetyl chymotrypsin as an intermediate and its subsequent preferential breakdown with ethanol rather than with water. The net result is of course an enzymatic transfer of acetyl from nitrophenyl acetate to ethanol, and the comparatively rapid breakdown of the acetate may be thought of as contingent upon the presence of the alcohol as a "cosubstrate" (2, 3).

It was of interest to see whether other substances acted in a manner analogous to ethanol, and a qualitative trial of substances in the laboratory, without any attempt at a thorough survey, showed that most of them did not markedly accelerate the decomposition of *p*-nitrophenyl acetate by chymotrypsin. It was observed that several substances besides cysteine and glutathione (4) decomposed *p*-nitrophenyl acetate at pH 6.2 without chymotrypsin, among them ethanolamine, histidine, and glycine, the last in fairly high concentration only. The substances which did accelerate the chymotryptic breakdown of *p*-nitrophenyl acetate without being active by themselves in this respect happened to be alcohols (in the cases tried), and included glycerol and monoacetin. It therefore seemed worth while to study the behavior of a series of the aliphatic monohydric alcohols as far as their solubility would permit. For practical reasons the series was limited to methanol, ethanol, the two propanols, the four butanols, *n*-pentanol, and *n*-hexanol.

It was found that the straight chain alcohols accelerated the breakdown of *p*-nitrophenyl acetate much more rapidly than their branched chain isomers. Moreover, the reaction rates observed with the straight chain compounds increased considerably with the length of the carbon chain (methanol being an exception, as it reacted faster than ethanol). Thus the reaction with *n*-hexanol was found to be at least fifteen times faster than

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with ethanol, and was easily followed at low molar concentrations at which the effect of ethanol could not be measured at all by our methods.

Materials and Methods

Materials—α-Chymotrypsin was prepared from α-chymotrypsinogen which had been crystallized five times. The active enzyme was crystallized twice, thereafter subsequently dialyzed against 0.001 M acetic acid, and finally lyophilized.

p-Nitrophenyl acetate was synthesized with acetyl chloride in the conventional manner, crystallized from aqueous ethanol, and recrystallized from petroleum ether. Commercial L-tyrosine ethyl ester was recrystallized before use. L-Tyrosine *n*-butyl ester (no longer available commercially), L-tyrosine isobutyl ester, and L-tyrosine *sec*-butyl ester were made from L-tyrosine and the respective alcohols containing 2 per cent dry HCl, the first two being made at room temperature.

L-Tyrosine *n*-butyl ester was obtained as the crystalline hydrochloride by precipitation with ethyl ether. It was then recrystallized from *n*-butyl alcohol by the addition of ether, and identified by elementary analysis, since no melting point could be found in the literature. Found, C 56.99, H 7.41, N 5.20; calculated, for $C_{13}H_{20}O_3NCl$, C 57.2, H 7.33, N 5.13. The hydrochloride melted at 166.0–167.5°, the free ester at 138–139°.[1]

L-Tyrosine isobutyl ester was also precipitated as the crystalline hydrochloride by the addition of ethyl ether to the reaction mixture. The hydrochloride was dissolved in water, and the free ester was isolated after making the solution alkaline with sodium carbonate. The free ester melted at 147–148.5° in agreement with the corrected value (5).

L-Tyrosine *sec*-butyl ester was recovered by evaporating the reaction mixture almost to dryness under reduced pressure and dissolving the residue in water. The aqueous solution was decolorized with charcoal, then neutralized with sodium carbonate. The crystalline precipitate of the free ester was removed and recrystallized from *sec*-butyl alcohol by the addition of petroleum ether. On analysis the following were found C 65.81, H 8.13, N 6.01; calculated, for $C_{13}H_{19}O_3N$, C 65.8, H 8.03, N 5.91. The ester melted at 118–121°.

The various alcohols and acetate esters used were the purest available commercially and were fractionated before use with a 25 cm. Vigreux column at atmospheric pressure.

Methods—The requisite amount of the alcohol in question was dissolved in 0.07 M phosphate, pH 6.20. To 9.6 ml. of this solution in a colorimeter tube (24 mm. diameter) there was first added 0.200 ml. of 0.125 M *p*-nitro-

[1] An old commercial catalogue gives 132–136° for the melting point of the free ester.

phenyl acetate dissolved in acetone, the solution being stirred well by the pipette during the addition. Immediately thereafter, and counted as zero time, 5.0 mg. of chymotrypsin, dissolved in 0.200 ml. of buffer, were added. The progress of the reaction, which consists in the liberation of nitrophenol, was observed by the increase in optical density at 402 mμ with a Coleman junior colorimeter.[2] Blanks consisting of everything except the enzyme were run simultaneously. The experiments were made in a constant temperature room at 25°. The progress of the reaction is illustrated for the case of *n*-butyl alcohol in Fig. 1. The other alcohols gave similar straight

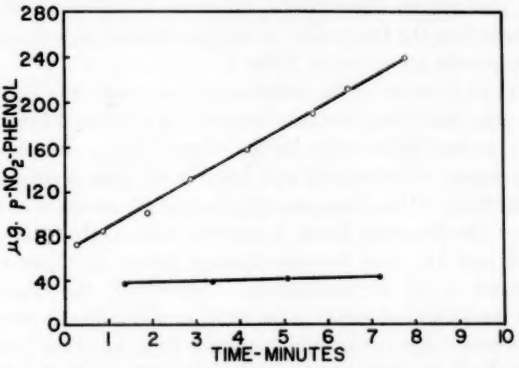

FIG. 1. Hydrolysis of *p*-nitrophenyl acetate in phosphate buffer (0.07 M, pH 6.20) containing *n*-butanol (0.68 M). ○, 0.50 mg. of chymotrypsin per ml.; ●, no enzyme. Calculated as microgram of nitrophenol per 10 ml.

lines which differed only in slope. The reaction was usually observed for about 20 minutes.

The formation as well as the disappearance of certain esters was also measured on occasion by the formation of hydroxamic acids in the manner described in a previous publication (1). Advantage was taken of the fact that all the esters reacted with hydroxylamine at pH 11.7, whereas reaction at pH 6.5 was practically confined in these tests to *p*-nitrophenyl acetate and acetyl chymotrypsin, the latter being present in relatively small amount.

The splitting of tyrosine ethyl ester by chymotrypsin was measured at 31° by titration with a micro burette at "constant" pH (pH 6.2) (6), 3 ml. of 0.100 M tyrosine ethyl ester being used.

[2] Since light absorption by *p*-nitrophenol is sensitive to small changes in pH, a new standard curve was determined for each new batch of buffer, and this solution was used within a few days.

Results

It was found impracticable to test all the alcohols at the same molar concentration because relatively high concentrations of the lower alcohols were required to obtain a decisive effect, while the higher alcohols were not soluble to that extent, or else they precipitated the enzyme. The experiments were therefore run in two series, higher alcohols at 0.048 M and lower alcohols at 0.68 M. The series overlapped in two instances, *n*-propanol and *n*-butanol, and a rough estimate of the relative rates of liberation of nitrophenol has been made, perhaps with dubious justification, by expressing all the rates in each series, respectively, in terms of the rate with *n*-butanol, and then connecting the two series by using *n*-butanol as a common denominator. The results are given in Table I.

If an exception is made of the behavior of methanol, the first member of the series, it is evident that increased length and decreased branching of the carbon chain favored higher rates for the release of nitrophenol. It appears from a comparison of *n*-butanol and isobutanol (also a primary alcohol) that any branching of the chain produced a marked decrease in the speed of the reaction. On the other hand, it may be noticed that the two secondary alcohols[3] and the one tertiary alcohol tested in these experiments scarcely reacted at all in comparison. Therefore, the distance of the branch from the hydroxyl group must be of considerable importance.

Ester Formation—It is reasonable to assume that the other alcohols tested here react in the same way as ethanol has been shown to do, namely with the formation of an acetate. The reaction was, however, tested with one other alcohol, *n*-propanol, by using the hydroxylamine test. It was found that, as the nitrophenyl acetate decreased, another ester was formed which reacted much more readily in alkaline hydroxylamine solutions than at pH 6.5. In Table II it is seen that, whereas the amount of *p*-nitrophenyl acetate decreased markedly, the total amount of esters in the system decreased much less so. Toward the end of the experiment a substantial quantity of ester was present, though nitrophenyl acetate had virtually disappeared. The decrease in total ester concentration during the experiment is most likely due to spontaneous hydrolysis of the *n*-propyl acetate formed. On testing the latter ester at 0.0025 M concentration in 0.07 M phosphate buffer at pH 6.2, it was found to decompose spontaneously at the rate of 0.43 μmole per hour per ml. The presence of chymotrypsin (0.50 mg. per ml.) did not change this rate. The results in Table II indicate a transfer reaction of considerable magnitude, and can account for the increased liberation of *p*-nitrophenol as determined by the color test in the presence of *n*-propanol.

[3] It may be noted that secondary butyl alcohol (methylethyl carbinol) possesses an asymmetric carbon atom. This might well have an influence on its behavior, and the question is receiving attention.

Behavior of Tyrosine Ethyl Ester—If the behavior of acetyl chymotrypsin is indicative of the mechanism whereby the hydrolysis of the "natural" substrates of that enzyme occurs, it seems reasonable to suppose that an amino

TABLE I

*Hydrolysis of p-Nitrophenyl Acetate in Presence of Various Alcohols**

Alcohol	Activity, γ *p*-nitrophenol liberated per min. per 10 ml.			
	Molarity	Observed	Increase due to alcohol	Approximate relative rates
		Ethanol = 1.0		
None	0	1.8		
Methanol	0.68	7.6	5.8	2.0
Ethanol	0.68	4.7	2.9	1.0
n-Propanol	0.68	7.6	5.8	2.0
n-Butanol	0.68	20.4	18.6	6.5
		n-Butanol = 6.5		
None	0	1.8		
n-Propanol	0.048	2.1	0.3	2
n-Butanol	0.048	3.0	1.2	6.5
n-Pentanol	0.048	4.0	2.2	12
1-Hexanol	<0.048†	4.6	2.8	15
		Ethanol = 1.0		
None	0	1.6		
Isopropanol	0.68	3.2	1.6	0.55
Isobutanol	0.68	9.7	8.1	2.8
sec-Butanol	0.68	3.8	2.2	0.76
tert-Butanol	0.68	2.5	0.9	0.3

* The system consisted initially of 0.20 ml. of 0.125 M *p*-nitrophenyl acetate, 0.5 mg. of chymotrypsin (in 0.2 ml. of buffer), the alcohol in amount to give the concentration shown, and 0.07 M phosphate buffer, pH 6.20, to make a final volume of 10 ml.

† This quantity did not dissolve completely.

acid residue replaces acetyl in the enzyme protein in the latter case, and that the aminoacylated protein is so quickly decomposed by water that its existence may not be detected. However, in view of the present findings, one of the higher alcohols used in this study might prove to be a good acceptor of the amino acid counterpart of acetyl, and thus lead to the formation of an amino acid ester of the alcohol used. The inhibition of chymotryptic esterolysis by alcohols is well known and has led to the opinion that

TABLE II

*Disappearance of Reactive Acetyl and Formation of Less Reactive Ester**

Micromoles of ester left per ml. of solution.†

Time	1.3 M *n*-propanol No enzyme		Difference	1.3 M *n*-propanol Enzme present		Difference
	pH 11.6	pH 6.7		pH 11.6	pH 6.7	
min.						
1.0				2.53	2.58	−0.05
9.0	2.55	2.50	0.05			
90				2.30	1.93	0.37
92	2.52	2.49	0.04			
180				2.17	1.12	1.05
182	2.58	2.53	0.05			
270				1.64	0.51	1.13
272	2.58	2.48	0.10			
395				1.25‡	0.015	1.24
397	2.40	2.44	0.04			

* Room temperature, about 26°.

† The system, as described in the text, contained per ml., at the start, 2.50 μmoles of *p*-nitrophenyl acetate, 1.3 mmoles of *n*-propyl alcohol, and 0.50 mg. of chymotrypsin.

‡ The decrease is probably due to the spontaneous hydrolysis of *n*-propyl acetate.

TABLE III

Apparent Inhibition of Hydrolysis of L-*Tyrosine Ethyl Ester by Various Alcohols at 31°**

Alcohol	Activity, m.eq. per min. per ml.	Relative inhibition ethanol = 1.0
None	0.00044	0
Methanol	0.00023	1.2
Ethanol	0.00027	1
n-Propanol	0.00019	1.5
n-Butanol	0.000080	2.1
Isopropyl	0.00036	0.5
Isobutyl	0.00018	1.5
sec-Butyl	0.00024	1.2
tert-Butyl	0.00040	0.2

* The composition of the reaction mixture (3.0 ml. in volume) was L-tyrosine ethyl ester 0.100 M, the alcohol 0.68 M, and α-chymotrypsin 0.021 mg. (introduced in 0.025 ml. of water). Titration was made at pH 6.2 ± 0.1 with 0.355 N NaOH for 10 to 30 minutes, depending upon the speed of the reaction. The rate was determined by the slope of the zero order curve obtained.

a transesterification might be at least in part responsible for it (7). That this is indeed the case has been definitely shown by the formation of tyrosine butyl ester when tyrosine ethyl ester was hydrolyzed by chymotrypsin in the presence of *n*-butyl alcohol.

The inhibition by several alcohols of the chymotryptic esterolysis of tyrosine ethyl ester is presented in Table III. It may be noted that these alcohols arrange themselves as inhibitiors of tyrosine ester hydrolysis in the

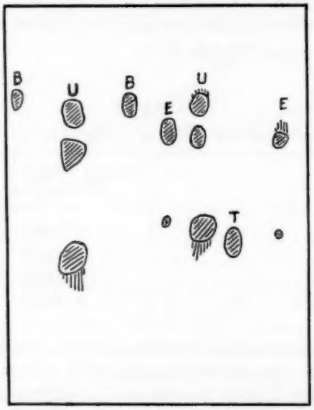

FIG. 2. Single ascent chromatogram of products from a system containing L-tyrosine ethyl ester 0.05 M, *n*-butanol 0.8 M, chymotrypsin 0.06 mg. per ml., after 60 minutes incubation at 25°, pH 6.2. Made on Whatman No. 4 paper (15 × 11.25 in.) with *n*-butanol-acetic acid-water (8) as the distributing liquid. Development was made with diazotized sulfanilamide (9). *T*, L-tyrosine; *E*, L-tyrosine ethyl ester; *B*, L-tyrosine *n*-butyl ester (all authentic samples); *U*, the incubated material (about 0.5 μmole of each of the known substances was employed in each case; for *U*, 10 μl. of solution were used).

same order (including methanol) as that of their reactivity with acetyl chymotrypsin (Table I).

It was found that the decrease in the measurable esterolysis of tyrosine ester in the presence of *n*-butanol was not entirely owing to the inhibition of the enzyme by the alcohol, but at least in part to its action as a transferase instead of a hydrolase. Considerable quantities of tyrosine butyl ester accumulated in the system; its presence could be easily demonstrated by chromatograms, an example of which is given in Fig. 2.[4] It is also

[4] Chymotrypsin, acting on tyrosine ethyl ester without butanol, gave only two spots, corresponding to free tyrosine and undecomposed ester. This eliminates the possibility that a peptide was formed whose rate of migration on paper was the same as that of tyrosine butyl ester. When chymotrypsin was added to a suspension of

evident that this particular transesterification did not occur because of the formation of a new ester that was not a good substrate in comparison with tyrosine ethyl ester. When the three butyl esters of tyrosine in our possession were tested separately, it was found (Table IV) that they were all hydrolyzed more rapidly than the ethyl ester. Moreover, when listed in the order of their relative rates of hydrolysis, this order is found to be the same as that observed for the respective accelerating effects of their corresponding alcohols on the decomposition of nitrophenyl acetate (Table I), and also for the respective inhibitory effects of these alcohols on the hydrolysis of tyrosine ethyl ester (Table III).

TABLE IV

Hydrolysis of Ester of L-*Tyrosine at 30°*

Ester	Initial velocity, m.eq. per min. per ml.	Relative rates
n-Butyl	0.00063	2.3
Isobutyl	0.00037	1.4
sec-Butyl	0.00033	1.2
Ethyl	0.00027	1

The composition of the reaction mixture (3.0 ml. in volume) was ester 0.014 M, and α-chymotrypsin 0.021 mg. (introduced in 0.025 ml. of water). Titration was made at pH 6.2 ± 0.1 with 0.355 N NaOH for 7 to 12 minutes, depending upon the speed. The initial rate was determined by extrapolation to zero time on a plot of rates against time.

The accumulation of the new ester may, however, be easily explained by the fact that its concentration in these systems is much less than that of the tyrosine ethyl ester from which it was formed.

On the other hand, the formation of new esters that are not easily hydrolyzed, if hydrolyzed at all, is shown by the formation of the acetic esters of the higher primary alcohols tested here. There is no evidence that any of these acetic esters are substrates for the enzyme. No measurable chymotryptic hydrolysis occurred with *n*-propyl acetate or methyl acetate (tested by the hydroxamic acid method), or with *n*-butyl acetate or isopropyl acetate (as tested by the titration method). It is curious that chymotrypsin may form an ester, *e.g.* methyl acetate, which it is not thereafter able to

tyrosine ethyl ester in 1.3 M *n*-butanol in water, a drop in the pH of the system occurred. Tyrosine butyl ester was isolated by extraction with either chloroform or *n*-butyl alcohol, and purified by recrystallization from aqueous methanol (m.p. 138–140°; mixed with authentic tyrosine butyl ester, m.p. 138–140°). The yield was 11 per cent. In the absence of enzyme, no drop in the pH was observed, and no tyrosine butyl ester was recovered.

split at a measurable velocity. It was found, however, that methyl acetate inhibited the chymotryptic hydrolysis of tyrosine ethyl ester, although in high concentration, and that this inhibition appeared to be competitive on the evidence of Lineweaver-Burk plots, which are presented in Fig. 3.

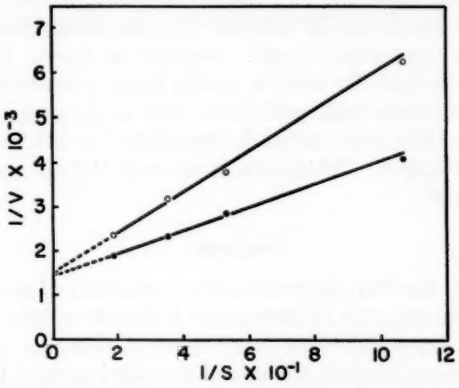

FIG. 3. Lineweaver-Burk plot, indicating that the hydrolysis of L-tyrosine ethyl ester is competitively inhibited by methyl acetate in high concentrations. Tyrosine ethyl ester; chymotrypsin, 0.0083 mg. per ml. ●, no methyl acetate; ○, methyl acetate, 0.8 M. *S*, initial molar substrate concentration, and *V*, initial rate expressed as millimoles of ester hydrolyzed per ml. per minute.

DISCUSSION

It would be difficult to explain the foregoing results on the basis of any simple collision theory. The length and shape of the alcoholic carbon chain and the position of the hydroxyl group in the alcohol molecule are definitely determining factors. One may therefore conclude that the alcohol as well as the acid group is attached to the enzyme molecule, and that a great difference exists between various alcohols in respect to the facility with which this attachment takes place. The enzyme exhibits the sort of "preference" that passes for "specificity" with many of the esterases. The attachment may be to an alcohol or to an ester, regardless of whether the latter is a substrate, as shown by the inhibitory effect of methyl acetate.

The formation of a new ester is obviously going to be more complete when it is not a good substrate. Therefore, the formation in considerable quantities of a new substance that is itself readily hydrolyzed must indicate that the affinity between the enzyme and the inhibitory alcohol is of no mean order. For this reason it would be incorrect to say that the inhibition of chymotrypsin by alcohols is nothing more than the occurrence of a transfer

reaction, but the evidence shows, nevertheless, that some of it is. A comprehensive kinetic study would doubtless be required to determine how much. This study the present authors have no intention of making.

Chymotrypsin, as an esterase, attacks substances of the type ROQ (10), where, for instance, R may be either nitrophenyl or methyl and Q may be acetyl or tyrosyl, respectively. That the enzyme forms an acetyl complex has been demonstrated by its isolation (11); an assumption is sometimes made that an analogous tyrosyl complex is merely less stable in water. However, that the enzyme readily forms complexes with various aliphatic alcohols seems quite probable in view of the present experiments. In so doing it exhibits about as much "specificity" as one may expect from this particular catalyst. Which side of the ester linkage really is the side of high specificity?

SUMMARY

Chymotrypsin has been shown to catalyze a transfer of acetyl from nitrophenyl acetate to aliphatic alcohols of low molecular weight. The enzyme exhibits a marked preference for certain types of alcohols. In a series concluding with *n*-hexyl alcohol, the rate of transfer increased markedly with the length of the straight carbon chain. Methanol was an exception. Where the chain was branched, the rate of transfer was much slower. In the case of the butyl alcohols, both primary alcohols reacted more rapidly than did the secondary, and the secondary more rapidly than the tertiary, which was almost inert.

All the alcohols tested inhibited the liberation of titratable acid (tyrosine) from L-tyrosine ethyl ester by chymotrypsin. *n*-Butyl alcohol was found to be a good inhibitor of this system, but at least part of this inhibition was found to be owing to a transesterification reaction leading to the formation of L-tyrosine butyl ester. When arranged in the order of their increasing inhibitory effect, the alcohols then occurred in the same sequence as when arranged in the order of increasing speed of acetyl transfer.

It may be noted that, whereas L-tyrosine butyl ester is a good substrate for chymotrypsin, methyl acetate and *n*-butyl acetate are not. Thus the enzyme appears to take part in the formation of some esters which it cannot split with measurable velocity.

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THE COENZYME CONTENT OF RABBIT MUSCLE D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE*

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In previous communications from this laboratory, the authors have reported the molecular weight of rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase (TDH[1]) to be 138,000 to 140,000 by light scattering (1) and 137,000 by sedimentation velocity and diffusion (2). Taylor (3) had previously reported the molecular weight to be 118,000 by sedimentation velocity and diffusion. This latter value was apparently confirmed for the crystalline enzyme by Velick, Hayes, and Harting (4), who recorded the increase in extinction at 340 mμ which occurred upon reduction of the bound DPN. These workers (4), using glyceraldehyde-3-phosphate as a substrate, found 1 mole of DPN per 58,000 gm. of protein, while Dandliker and Fox (1), using glyceraldehyde as substrate, found 1 mole of coenzyme per 58,500 gm. of enzyme.

However, in interpreting these studies on the coenzyme content of the crystalline enzyme, three assumptions were involved: first, the crystalline enzyme preparation has an integral number of moles of coenzyme per mole of apoenzyme; second, the increase in absorption at 340 mμ for the reduction of DPN is the same for bound coenzyme as it is for the coenzyme free in solution; and third, the enzyme is able to reduce all of the bound coenzyme. Since the molecular weight obtained by physical methods in this laboratory (138,000) is not a multiple of the minimal molecular weight as determined from the enzymatic reduction experiments (58,000), it seemed advisable to examine the assumptions made in studying the DPN content of the enzyme.

In considering the first assumption, it should be pointed out that, in

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[1] The following abbreviations are used: DPN for diphosphopyridine nucleotide; TEA for triethanolamine; TDH for D-glyceraldehyde-3-phosphate dehydrogenase; EDTA for ethylenediaminetetraacetic acid.

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general, there is no *a priori* justification for assuming that the molar ratio of components in a crystal should be an integral number, although non-integral ratios, *e.g.* those in certain crystals containing water of hydration, are somewhat uncommon. The extent to which the DPN is concerned in the crystalline structure of the enzyme is not known, but that the coenzyme might be involved in some way is suggested by the report of Taylor *et al.* (5) that the apoenzyme did not crystallize in the absence of the coenzyme.

The finding of Theorell and Bonnichsen (6) is of interest in connection with the second assumption. These workers found that the absorption peak due to the reduction of bound DPN on horse liver alcohol dehydrogenase was shifted and lowered when compared to the reduction of an equal amount of free DPN. That a similar phenomenon might occur when the coenzyme is bound to TDH remains a possibility.

The third assumption appears questionable in view of the finding by Cori, Velick, and Cori (7) that the bound DPN on the enzyme was only 70 per cent reduced in the presence of arsenate and stoichiometric amounts of substrate, the arsenate being added to drive the reaction to completion. Furthermore, when the presence of bound DPN was first demonstrated by Taylor *et al.* (5), only 60 per cent as much coenzyme could be found by enzymatic reduction as could be found by analysis for the various chemical constituents, adenine, nicotinamide, phosphate, and ribose. It may be noted that the DPN content determined by reducing the bound coenzyme would be too low if either the second or the third assumption was invalid. These findings all seemed to indicate the advisability of determining the DPN content of the complex by some independent means.

Perhaps the best method for studying the coenzyme content of the complex is to remove all of the DPN and reconstitute the complex with known amounts of apoenzyme and coenzyme. However, for this method to be quantitative the extent of removal of the coenzyme must be known exactly, and some means must be available for determining when the complex is fully reconstituted. The method used heretofore to remove the coenzyme from the apoenzyme has been a batch process in which the complex is stirred with a large excess of charcoal for a few minutes, the charcoal then being removed by centrifugation or filtration. This method suffers from the inherent disadvantage that the removal of the coenzyme is characteristic of a single distribution of the coenzyme between the bound and adsorbed phases for each application of the process.

In the following discussion the use of a charcoal column to remove the coenzyme will be described. This technique has the theoretical advantage of greater efficiency than a batch process, and was found to be effective in producing the maximal possible removal of the coenzyme by charcoal.

Some of the properties of the apoenzyme thus produced will be described, and the result of the quantitative titration of the apoenzyme with the coenzyme will be presented, this latter process being followed by the increase in absorption in the 300 to 400 mμ region which arises when the DPN is bound to the apoenzyme (8).

EXPERIMENTAL

Preparation of Enzyme—Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was prepared as described previously (1) and stored at 0° as the crystalline suspension in approximately two-thirds saturated ammonium sulfate and 2.0 mM EDTA.

Coenzyme—The DPN used for the enzymatic assays and the binding studies was Pabst lot No. 308. This material, prepared from yeast, is

TABLE I

Optical Extinction Data for DPN Extinction Coefficients, Cm.² Mole⁻¹ × 10⁻⁶

Source or literary reference	$\lambda_{m\mu}$		
	260	280	340
Pabst lot No. 308 (this work)	19.7*	4.38	0.021
Heat denaturation of apoenzyme (this work)	20.3	4.35	0.112
Yeast (4)	19.6	4.38	
Kornberg and Horecker (9)	18.0		

* The discrepancy between this value and that given by Kornberg and Horecker (9) may be due to the presence of the α isomer of DPN (10).

approximately 95 per cent oxidized DPN on a dry weight basis.[2] The extinction coefficients at 260, 280, and 340 mμ are given in Table I, along with the corresponding values for DPN derived from the TDH-DPN complex by heat denaturation of the apoenzyme. Also in Table I are the values for the extinction coefficients reported by Velick *et al.* (4) for a DPN preparation of 94 to 97 per cent purity, and the value given by Kornberg and Horecker (9). In this work, DPN concentrations were determined by reduction of the coenzyme with excess glyceraldehyde and arsenate in the presence of TDH, followed by measurement of the increase in absorption at 340 mμ arising from reduced DPN. The extinction coefficient used for the reduced coenzyme was 6.25×10^6 cm.² mole⁻¹, which is an average of the values reported by Horecker and Kornberg (11) and Ohlmeyer (12). In a comparison experiment, identical DPN assays were obtained by using either glyceraldehyde-3-phosphate or glyceraldehyde as substrate.

[2] Manufacturer's specifications.

Charcoal—The charcoal was Norit A (Pfanstiehl Laboratories), which was used without further treatment.

Filter Pulp—The filter pulp used was the dispersed material (No. 289) manufactured by Schleicher and Schüll.

Enzymatic Assay—The enzymatic assay system used in this work was that described by Beisenherz *et al.* (13), except that glyceraldehyde (16.7 mM) was used as substrate at pH 7.5. Under these conditions, the reduction of DPN was linear with time for several minutes, and the initial rate of reduction of coenzyme was proportional to enzyme concentration.

Absorption of Protein in Ultraviolet Region—The absorption spectra reported in this paper were determined either in a Beckman DU spectrophotometer or in a Cary recording spectrophotometer. The Beckman instrument was used whenever precise determinations of extinctions were required as well as for kinetic work.

Extinction Coefficients—All protein extinction coefficients reported in this paper were determined from the dry weight of protein in solutions of measured optical absorption. After equilibrating the protein against the appropriate buffer, measured portions were put into weighing bottles and the solution lyophilized in an Abderhalden drying pistol. The lyophilized protein was then dried overnight at 100° *in vacuo* over P_2O_5 in the Abderhalden pistol, cooled, and weighed. The process was repeated for 12 to 24 hour intervals until the protein no longer lost weight. A blank correction for the buffer alone was made in each case.

Charcoal Columns—The theoretical advantage of the use of charcoal columns for the removal of the coenzyme has been mentioned previously, but it has been found that columns composed solely of charcoal have low flow rates, resulting in long contact times which cause the protein from the column to be unstable. Since activated charcoal contains divalent metal ions, it is possible that the instability noted after prolonged association with charcoal is due to oxidation of sulfhydryl groups on the protein catalyzed by a heavy metal ion (14, 15). In order to attain higher flow rates, various supporting media were tried, and it was found that equal portions of charcoal and coarse filter pulp produced columns which were entirely satisfactory. In practice, the charcoal and pulp are mixed together in distilled water for several minutes and then gently packed into a column with a footed glass rod. A mixture of 1.25 gm. of charcoal and 1.25 gm. of filter pulp, when packed in a column 0.8 × 20 cm., gives a flow rate of 8 ml. per hour under a head of 4 to 6 inches of solvent, with a void volume of 8 ml. in the column. This column is adequate to give maximal removal of DPN from 3 ml. of a 5 per cent enzyme solution, the yield off the column being about 4 ml. of a 2.5 to 3.0 per cent solution. For the charcoal treatment, a portion of centrifuged crystals was dissolved in

water; no additional EDTA was added over that already present in the occluded mother liquor. The column was jacketed and kept at 1° with provisions for performing the experiment under a nitrogen atmosphere.

Phosphate Analysis—Phosphate analyses were carried out according to the method of King (16). Optical densities were measured at 660 mμ in a Beckman DU spectrophotometer by using 1 or 5 cm. path lengths.

Heat Denaturation—In order to determine the amount of DPN in the enzyme-DPN complex, the apoenzyme was removed by heat denaturation. Enzyme solution in a tightly stoppered test-tube was immersed in water at 100° for 1.5 minutes. The tube was then cooled in ice water, unstoppered, and the protein gel broken up and removed by centrifugation. No loss of solvent was noted when this technique was used. Dr. F. M. Huennekens of this department has subjected the supernatant solution prepared by heat denaturation to paper chromatography and found two nucleotide spots, both of which assayed as DPN. The solvent system was isobutyric acid, concentrated ammonium hydroxide, and water, in a volume ratio of 66:1:33. Separation of the DPN in this case probably is due to the acidity of the solvent. (This effect was studied by Chaykin (17), who found two spots for DPN below pH 4.75 and only one spot at higher pH values.) Chromatograms of DPN from the enzyme carried out in acetone-water (2:1 by volume) or in a mixture of 600 gm. of ammonium sulfate, 1 liter of sodium phosphate buffer (0.1 M, pH 6.8), and 20 ml. of *n*-propanol resulted in a main spot similar to that for commercial DPN in respect to both rate of migration and appearance under ultraviolet light. The R_F values obtained in these two solvents were 0.10 and 0.31, respectively, for commercial DPN, and 0.09 and 0.28 for DPN from TDH. In both solvents, two other faint spots were detected in the DPN from the enzyme, but neither of these seems to be due to the presence of reduced DPN.

Addition of DPN to Apoenzyme—The apoenzyme for these experiments was prepared fresh on a charcoal column. If the ultraviolet absorption indicated that all of the DPN had been removed (see below), portions of apoenzyme and DPN solutions were mixed with distilled water to make the volume up to 3 ml. in a Beckman cell. The binding of the coenzyme was followed by the increase in extinction at 340 mμ (8).

RESULTS AND DISCUSSION

Enzymatic Activity—The enzyme, acting upon glyceraldehyde at pH 7.5 under conditions of zero order kinetics, has been found to have a turnover number of 6.0 to 6.5 moles of DPN per minute per 10^5 gm. of enzyme. None of the techniques which have been used in this work, with the exception of those deliberately used to denature the protein, has effected any change in the activity of the enzyme on this substrate; this finding is

especially noteworthy in the case of the apoenzyme from the charcoal column.

Absorption of Protein in Ultraviolet Region—It has been reported (4) that the absorptions of DPN and apoenzyme are additive in the region from around 260 to 280 mμ, so that the extinction of the apoenzyme should be obtainable by simply subtracting the contribution of DPN from the absorption of the complex. Both ultraviolet absorption and dry weight determinations were run on one portion of TDH solution. Another portion was subjected to heat denaturation, and the supernatant solution was analyzed for DPN by enzymatic reduction. The calculations were carried out as indicated above, and the values of $E_{280} = 0.890$ and $E_{260} = 0.483$ cm.^{2} mg.$^{-1}$ were obtained for the apoenzyme. These two values are significantly lower than those reported previously by Velick *et al.* (4), $E_{280} = 0.98$ and $E_{260} = 0.535$; the ratio of the apoenzyme extinction at 280 mμ to that at 260 mμ calculated by assuming mere additivity is also too low. This ratio, E_{280}/E_{260}, hereafter referred to as R, is a sensitive measure of the DPN content of the complex, since these two wave-lengths represent the maximal absorption of the apoenzyme and the coenzyme, respectively. Since the 260 mμ extinction is in the denominator and represents the DPN content, it can be seen that the lower the DPN content, the higher the ratio. From the charcoal column work (see below) it has been found that the maximal ratio obtainable by removal of the coenzyme from the apoenzyme is 1.92, and it is to be expected that the extinctions at 280 and 260 mμ calculated for the apoenzyme should have at least this ratio. Since the extinctions calculated on the basis of additivity of absorption gave R values which were too low, it appeared that either the assumption of additivity was wrong or the heat denaturation of the protein resulted in the loss of some of the DPN, so that the quantity of coenzyme assayed was too low.

In order to determine which of these alternatives might offer the correct explanation, the extinction coefficient, based on protein dry weight, was determined for the charcoal-prepared apoenzyme. The average results for two determinations which agreed closely were $E_{280} = 0.829$ and $E_{260} = 0.435$ cm.^{2} mg.$^{-1}$, the ratio R being 1.92. These results indicate that there is a slight increase in extinction in the 260 to 300 mμ region when the apoenzyme binds the coenzyme. That this is actually the case was demonstrated by the following experiment: the extinctions and concentrations of a DPN solution and of a charcoal-prepared apoenzyme solution were determined and measured portions of the two solutions mixed. The concentrations of the protein and the DPN were such that all of the coenzyme was bound by the apoenzyme. The results of this experiment are shown in Fig. 1. By using the data of Fig. 1, the increase in absorption due to

the binding of DPN to the apoenzyme may be calculated. Since the DPN is present in less than equivalent amounts, the observed change in extinction must be divided by the DPN concentration and not by the protein concentration. The calculated values of Δ log (I_0/I) per cm. at 260 and 280 mμ, due to the binding, are 2.40 and 3.60, respectively, per

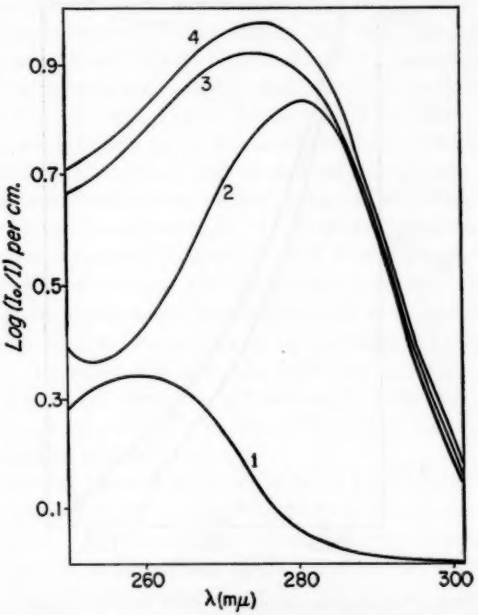

FIG. 1. Absorption spectra for the addition of DPN to the apoenzyme. Curve 1 represents 0.0154 mM of DPN, Curve 2, 1 mg. of apoenzyme per ml. Curve 3 is the sum of Curves 1 and 2, and Curve 4 is the experimentally observed curve for a solution 0.0154 mM in DPN and containing 1 mg. of apoenzyme per ml. This amount of DPN is about 70 per cent of the total binding capacity of the apoenzyme. Thus the difference between Curves 3 and 4 would be even greater if a stoichiometric amount of DPN had been added.

micromole of DPN bound per ml. By using these data, an empirical curve for the variation of the ratio R with varying amounts of DPN bound per mg. of enzyme per ml. was constructed as indicated in Fig. 2, upper curve. It will be noted that the curve has a break at 0.0216 μmole of DPN per mg. of enzyme per ml. Since this has been found to be the maximal number of moles of DPN bound in this study, the curve beyond this point has been calculated by adding the absorption of free DPN to the absorption of the completely reconstituted complex.

Three types of experimental evidence in support of the constructed curve have been obtained. The squares in Fig. 2 represent data on the number of micromoles of DPN per mg. of enzyme for various crystalline

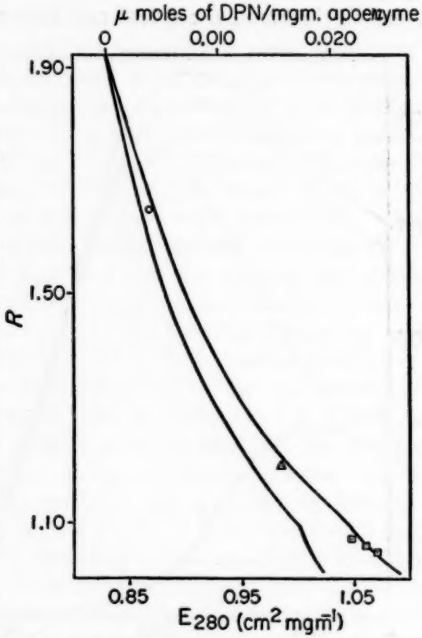

FIG. 2. The effect of varying the amount of DPN bound to the apoenzyme on the ultraviolet absorption of the complex. R is the ratio, E_{280}/E_{260}. The upper curve is the number of micromoles of DPN per mg. of apoenzyme per ml., while the lower curve represents the extinction coefficient at 280 mμ for any given R. The composition of the solution represented by a progression along the abscissa varies from pure apoenzyme to a completely reconstituted complex mixed with excess DPN. The break in each curve appears at the point of maximal binding. The symbols are to be associated with the upper curve and represent corroborative data from separate experiments (see the text).

preparations of the enzyme, as determined by heat denaturation of the apoenzyme and enzymatic reduction of the free DPN. The triangle (Fig. 2) is an experimentally determined point for the addition of DPN to an apoenzyme solution, the coenzyme being a Pabst DPN solution of known concentration, and the circle represents the result of only partial removal of DPN by charcoal. The DPN for the latter value was determined by the extinction of the heat-denatured solution at 260 mμ, followed by en-

zymatic assay, both of which agreed within 1 per cent. The phosphate analysis of the enzyme solution partially saturated with DPN was about 5 per cent higher than would be calculated from the DPN concentration, but this has been the usual finding with the enzyme from charcoal treatment. As a final check on the accuracy of the above determinations of the apoenzyme extinction and the increase due to the DPN binding, the extinction of the enzyme-$(DPN)_2$ complex has been calculated by using the molecular weight of 140,000 obtained in this laboratory from light scattering and sedimentation velocity and diffusion (1, 2). The values obtained were $E_{280} = 1.002$, $E_{260} = 0.922$, and $R = 1.09$. These values are in close agreement with the extinctions for the recrystallized enzyme, both as reported previously (4) and as determined directly in this work. The variations in the extinctions of the recrystallized complex are probably due to the small variations in the DPN content discussed previously.

Criteria for Completeness of Removal of DPN from Apoenzyme by Charcoal Treatment—Since the primary interest in removing the DPN from the apoenzyme was to obtain quantitative evidence as to the coenzyme content of the complex, it was necessary to be sure that the removal of DPN was complete. To attain this end, the following criteria were applied to the charcoal-treated enzyme: the achievement of the maximal possible extinction ratio, R, the absence of detectable DPN, and the absence of phosphate indicative of DPN.

That the maximal possible removal of DPN by charcoal had been obtained was demonstrated by passing a solution of the enzyme through two successive columns. Several samples were taken from the first and second columns; all samples gave a value of $R = 1.92$, demonstrating that the maximal removal had been attained by the first column. Solutions of the apoenzyme from the column were subjected to heat denaturation, and the supernatant solutions were examined spectrophotometrically. The remaining absorption, while not typical of the absorption of DPN, would have corresponded at 260 $m\mu$ to less than 0.5 per cent of the original amount of coenzyme in the complex. Phosphate analyses showed a slight amount of total phosphate in the apoenzyme amounting to about 6 per cent of the original content. Half of this phosphate was dialyzable and assayed as inorganic phosphate, with somewhat less than 3 per cent of the original phosphate remaining. In view of these results, it has been concluded that the charcoal column treatment of the complex as used in this work has completely removed the DPN from the apoenzyme.

Addition of DPN to Apoenzyme—The results of the titration of the enzyme with DPN are shown in Fig. 3. The rising portion of the curve was obtained by the least squares treatment of the results of six additions of DPN to the apoenzyme, whereas the level portion was obtained by averag-

ing the results of seven additions. The intersection of the two sections of the curve is at 0.0217 μmole of DPN per mg. of enzyme, which corresponds to 1 mole of DPN per 46,000 gm. of enzyme. This is equivalent to 3 moles of DPN per 138,000 gm. of the enzyme, the molecular weight thus calculated being in excellent agreement with the physical studies performed in this laboratory.

Enzymatic Reduction of Bound DPN—The usual method of enzymatically assaying bound DPN on the enzyme has been to add substrate to the

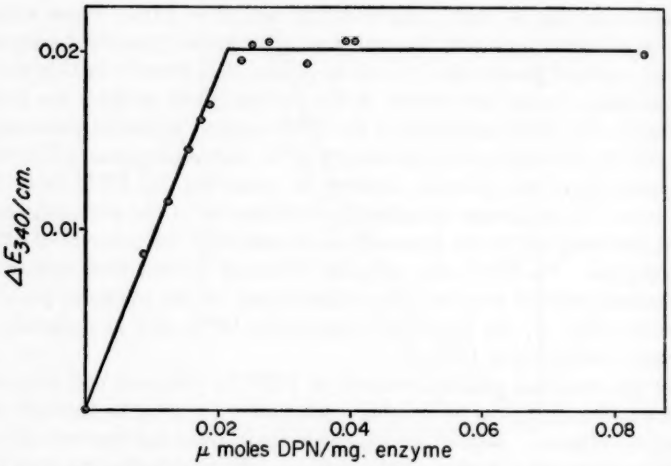

FIG. 3. The titration of apoenzyme with DPN utilizing the increase in absorption at 340 mμ. All the points represent solutions containing 1 mg. of protein per ml. The sudden break in the curve occurs at 0.0217 μmole of DPN per mg. of apoenzyme. The equation for the rising portion of the curve is ΔE_{340} per cm. = 0.925 × micromoles of DPN per mg. of enzyme, while the horizontal portion is $\Delta E_{340} = 0.0201$.

complex and to observe the change in the 340 mμ absorption (1, 4). However, the technique used in these instances has involved the use of the crystalline complex which may vary somewhat in DPN content. Since, in this work, the complex has been reconstituted with DPN solutions of known concentration, it was possible to know the exact amount of bound coenzyme. Apoenzyme and DPN were mixed with 50 mM TEA buffer, pH 7.5, and 6 mM in arsenate, so that the final concentrations were total protein 4.7 mg. per ml., total DPN (presumably all bound) 0.041 mM. It may be noted that this amount of DPN is 40 per cent of the maximal amount which could be bound to the protein present. 0.1 ml. of 0.5 M glyceraldehyde was added to start the reaction (total volume of solution,

3.0 ml.). Additional DPN was added after the initial reaction had stopped, whereupon the absorption at 340 mμ continued to increase beyond that expected from the binding of the added DPN, which indicates that the enzyme was still active. The initial increase in absorption corresponded to only 0.0285 mM DPN; *i.e.*, to only 70 per cent of the coenzyme originally added to the solution. In view of the findings of Cori *et al.* (7) previously referred to, we believe that our results indicate partial reduction and not a lowered extinction coefficient for DPN.

Because the enzymatic reduction of the bound coenzyme is not complete, it can be seen that the results of such an experiment cannot be adduced as proof of the molecular weight unless the percentage reduction is also known. Another objection to the previous determinations of the bound DPN by this method is that they have been made on the recrystallized apoenzyme-DPN complex. As has been mentioned previously, it cannot be rigorously

TABLE II

DPN Content of Crystalline Enzyme Preparations*

Preparation No.	Times recrystallized	Moles DPN / 10^5 gm. protein	R
7	8	2.2	1.07
8	4	1.92	1.14
9	7	2.35	1.05_5

* Measured by heat denaturation of the apoenzyme, followed by enzymatic reduction of the supernatant solution.

assumed that the crystalline enzyme will bind an integral number of moles of DPN, although it would appear reasonable in this case. In fact, the quantity of DPN in the recrystallized complex does show some slight variation, as presented in Table II. It will be noted that Preparation 8 has significantly less DPN than Preparation 7 or 9, in spite of the fact that Preparation 8 had been recrystallized fewer times. The DPN content of Preparation 8 was slightly less than would be expected for the enzyme-$(DPN)_2$ complex, and the crystalline enzyme was still losing small amounts of the coenzyme upon successive recrystallizations. In the case of Preparations 7 and 9, the loss of DPN was low upon successive recrystallizations. However, both of these preparations had considerably more DPN than a stoichiometric amount of the coenzyme. The authors, therefore, believe that the analysis of the coenzyme content of the crystalline enzyme is not a reliable quantitative criterion for a minimal molecular weight.

Evidence from Literature for Molecular Weight of 140,000—Additional evidence from the literature for the molecular weight reported in this pa-

per has come from two sources, the valine end-group analysis by Velick and Udenfriend (18) and the glutathione analysis by Krimsky and Racker (19). The reported value for the valine amino end-groups was 1.7 per mole, for a molecular weight of 118,000. This number, however, corresponds to an integral number of 2.0 per 139,000 in good agreement with the value from this laboratory. The glutathione analysis was 1.6 per mole, presumably for the molecular weight of 118,000 to 120,000 previously reported in the literature (3). This value corresponds to 1.87 moles of glutathione per 140,000, which is a closer approximation to an integral number. However, the glutathione is removed from the enzyme by tryptic digestion, and there is no evidence to show whether all of the glutathione had been removed and subsequently recovered.

SUMMARY

Quantitative studies have been made on the amount of DPN bound by D-glyceraldehyde-3-phosphate dehydrogenase. The methods applied to this problem have included the removal of the coenzyme from the protein by a charcoal column technique. The recombination of the coenzyme with the apoenzyme was studied quantitatively by the increase in absorption at 340 mμ. The results indicate that the enzyme binds 1 mole of DPN per 46,000 gm. of protein or 3 moles per 138,000. This latter figure is in good agreement with the molecular weight obtained by physical measurements. Evidence from the literature is presented which tends to corroborate these findings. The extinction coefficients of the apoenzyme have been found to be 0.829 and 0.435 $cm.^2$ $mg.^{-1}$ at 280 and 260 mμ, respectively. The DPN content of the complex has been determined by heat denaturation of the apoenzyme, followed by enzymatic reduction of the free DPN. Direct enzymatic reduction of the bound DPN appears to result in only 70 per cent reaction.

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ON THE MECHANISM OF AN ANAEROBIC EXCHANGE REACTION CATALYZED BY SUCCINIC DEHYDROGENASE PREPARATIONS*

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Attempts in this laboratory to study with deuterium the possible mechanism of an anaerobic hydrogen transfer reaction from reduced diphosphopyridine nucleotide to fumarate, as catalyzed by heart particle preparations (1), necessitated an investigation of the possibility that these preparations, which contain succinic dehydrogenase, also catalyzed an anaerobic exchange reaction described by Weinmann *et al.* (2). These workers observed that, when dideuteriosuccinate was incubated anaerobically with a rabbit kidney succinic dehydrogenase preparation, there resulted a significant exchange of the hydrogen atoms of the medium with the deuterium atoms of the dideuteriosuccinate. They also noted that this rate of exchange, although not influenced by the presence of methylene blue, was roughly equivalent to the rate of succinate oxidation in the presence of a suitable acceptor such as methylene blue (MB). This correspondence between the rates of anaerobic exchange and oxidation was interpreted as lending support to the hypothesis that the same enzyme, namely succinic dehydrogenase, catalyzed both of the following reactions: (1) oxidation of succinate and (2) hydrogen exchange.

(1) $$HOOC{-}CH_2{-}CH_2{-}COOH + MB \rightarrow HOOC{-}CH{=}CH{-}COOH + MBH_2$$

(2) $$HOOC{-}\underset{D}{CH}{-}\underset{D}{CH}COOH + 2H^+ \rightarrow HOOC{-}\underset{H}{CH}{-}\underset{H}{CH}{-}COOH + 2D^+$$

The results presented in this paper show that, when normal succinate was incubated anaerobically with a heart particle preparation in a medium containing D_2O, the exchange reaction was exceedingly slow compared with the rate of aerobic oxidation. The addition of fumarate greatly enhanced

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the rate of deuterium incorporation into succinate under anaerobic conditions. Thus the exchange reaction studied here appears to be not a result of an increased rate of dissociation of the C—H bonds of succinate as suggested in Equation 2, but rather a reflection of the oxidation of succinate

TABLE I

Anaerobic Deuterium Incorporation into Succinate, Fumarate, and Malate

Series No.*	Experiment No.	Acids incubated	Per cent D_2O in medium	Incubation period in hrs.	Per cent succinate oxidizable aerobically†	Acids isolated‡	Dilution factors	Deuterium content	
								Atom per cent excess§	Atom per molecule‖
I	1	Succinate	83.3	10	129	Succinate	2.0	0.119	0.0143
	2	" + fumarate	81.3	5	79	"	5.0	0.362	0.109
						Fumarate	2.0	0.017	0.0014
						Malate	10.0	0.257	0.720
	3	Succinate + fumarate	81.3	10	159	Succinate	5.1	0.786	0.241
						Fumarate	2.5	0.028	0.0028
						Malate	7.4	0.321	0.666
II	1	Succinate	87.5	8	334	Succinate	2.0	0.258	0.0310
						Fumarate¶	2.0	0.069	0.0055
						Malate¶	No dilution	0.043	0.0077
	2	Succinate + fumarate	85.8	2	67	Succinate	3.1	0.490	0.0996
						Fumarate	3.0	0.069	0.0083
						Malate	10.4	0.424	0.794
	3	Succinate + fumarate	85.8	4	134	Succinate	6.1	0.613	0.224
						Fumarate	6.0	0.033	0.0079
						Malate	9.7	0.468	0.817
	4	Succinate + fumarate	85.8	8	268	Succinate	11.9	0.644	0.460
						Fumarate	10.0	0.047	0.0188
						Malate	12.1	0.365	0.779
	5	Fumarate	91.8	8		Succinate¶	1.2	0.053	0.0038
						Fumarate	5.0	0.068	0.0136
						Malate	10.4	0.463	0.867
	6**	Succinate	87.5	8		Succinate	1.2	0.068	0.0048
	7**	Succinate + fumarate	85.8	8		"	1.2	0.061	0.0044
						Fumarate	3.0	0.025	0.0030
	8**	Fumarate	91.8	8		Fumarate	3.0	0.099	0.0119
	9**	Malate	87.5	8		Malate	No dilution	0.038	0.0068

TABLE I—*Concluded*

Series No.*	Experiment No.	Acids incubated	Per cent D_2O in medium	Incubation period in hrs.	Per cent succinate oxidizable aerobically†	Acids isolated‡	Dilution factors	Deuterium content	
								Atom per cent excess§	Atom per molecule‖
III	1	Succinate	87.5	20	258	Succinate	3.2	0.196	0.0376
	2	" + fumarate	85.8	5	31.5	"	5.7	0.405	0.138
						Fumarate	10.5	0.141	0.059
						Malate	8.6	0.336	0.809
	3	Succinate + fumarate	85.8	10	63	Succinate	7.8	0.563	0.263
						Fumarate	11.0	0.234	0.103
						Malate	8.7	0.346	0.843
	4	Succinate + fumarate	85.8	20	126	Succinate	16.7	0.534	0.535
						Fumarate	10.9	0.530	0.231
						Malate	11.8	0.313	1.034
	5	Fumarate	85.8	20		Succinate¶	1.6	0.064	0.0061
						Fumarate	9.0	0.217	0.078
						Malate	8.3	0.349	0.811
IV		Succinate + fumarate	78.8	20	206	Succinate	10.4	1.019	0.636
						Fumarate	3.1	2.040	0.253

* Each series represents one enzyme preparation with which all the experiments were carried out simultaneously.

† See the text, p. 1022.

‡ Malates in Series I and II isolated as cinchonine salt and in Series III as diphenacyl ester.

§ Experimental values.

‖ Values corrected for dilution and calculated on the basis that 1 atom of deuterium per molecule of succinic acid, fumaric acid, cinchonine malate, and diphenacyl malate would correspond to a value of 16.7, 25.0, 3.57, and 5.55 atoms per cent excess, respectively.

¶ Added as carrier at the end of the experiment. 1 mmole each for succinate and fumarate and 0.5 mmole for malate.

** Boiled enzyme.

(Equation 1) by a natural carrier in such a manner that the hydrogens of succinate are directly or indirectly donated to the medium (see "Discussion," Equations 5 and 6). This possibility was also considered by Weinmann *et al.* (2) and earlier by Erlenmeyer *et al.* (3). According to this view, the addition of unlabeled fumarate promotes the back-reaction by which the deuterium from the heavy water medium becomes incorporated into the succinate. This effect of fumarate might conceivably have in-

volved a prior incorporation of deuterium into fumarate if the fumarase present in our preparations had been non-stereospecific with respect to the methylene hydrogens of *l*-malate. However, experiments are reported herein which demonstrate the stereospecific behavior of fumarase, in agreement with similar results obtained by Fisher *et al.* (4).

On the assumption that the anaerobic exchange reaction is catalyzed by a single enzyme and that this enzyme is involved in the aerobic oxidation of succinate to fumarate, the data further suggest that all four methylene hydrogens of succinate were labeled as a result of the anaerobic exchange reaction. This result will be considered in terms of the stereospecificity of succinic dehydrogenase. A preliminary account of this work has appeared (5).

Methods

Enzyme Preparations, Assays, and Anaerobic Incubations—The heart muscle preparations were isolated from horse heart according to the method of Keilin and Hartree, as described by Slater (6). The anaerobic reaction mixtures contained 2.44 mmoles of potassium phosphate buffer, pH 7.29, 11.0 mg. of cytochrome *c*, 1.0 ml. of enzyme preparation, and 1 mmole of sodium succinate or potassium fumarate or 1 mmole each of succinate and fumarate, in a total volume of 16.5 ml. at the indicated per cent D_2O concentration by volume.[1] The succinate was tipped from the side arm of a large Thunberg tube after flushing the remainder of the reaction mixture in the main compartment with nitrogen gas and evacuating the tubes for 5 to 8 minutes with an oil pump. Incubations were carried out in a water bath at 37°. The corresponding rates of succinate oxidation were determined by following manometrically the oxygen uptake under aerobic conditions identical otherwise with those prevailing in the experimental anaerobic incubations. These rates are expressed (Table I) as per cent of the total succinate added which could be oxidized in the experimental period. Fumarase activity was determined under aerobic conditions by measuring the rate of loss in absorption at 240 mμ in a Beckman DU quartz spectrophotometer (7), and was checked by measurements of total malate isolated at the end of the anaerobic reactions.

Separation, Isolation, Dilution, and Purification of Acids—Reactions were stopped at the indicated time by acidification with concentrated H_2SO_4 to give a final concentration of approximately 2 N H_2SO_4. After filtration and washing the precipitate with 2.5 ml. of H_2O, the combined supernatant fluids were generally mixed with two weights of ether-washed Johns-Manville Celite No. 535. The acids were subsequently recovered by continuous ether extraction for 16 to 20 hours. After removal of the ether

[1] The heavy water used in these experiments was obtained on allocation from the United States Atomic Energy Commission.

under a vacuum in the presence of 5 ml. of water, the aqueous solution was adjusted to pH 8.0 to 8.5 with KOH and passed through Dowex 1-formate columns, and the acids were separated by a slight modification of the method described by Busch *et al.* (8).[2] Subsequent to desiccation, the fractions representing individual peaks were combined by dissolving in H_2O. The identity of each peak was determined by its position of emergence from the Dowex 1-formate columns (titration with NaOH) and by ascending paper chromatography in the butanol-propionic acid-H_2O solvent described by Benson *et al.* (9), and then spraying the dried paper with bromophenol blue (10). Prior to isotope dilution by addition of carrier, each acid was quantitatively determined as presently described. Succinate was determined manometrically by measuring the total oxygen uptake upon incubation with the succinic oxidase preparation from heart. Fumarate was measured by determining the optical density at 240 mμ in the Beckman spectrophotometer (7). Malate was determined fluorometrically by the method of Speck as described by Loewus *et al.* (11). After appropriate isotope dilutions with the respective acids, succinate and fumarate were isolated as the free acids and further purified by vacuum sublimation. Malate in some experiments was isolated as the cinchonine salt (12, 13), which was recrystallized once from methanol-acetone and finally from water-acetone. In other experiments malate was isolated as the diphenacyl ester (11) and recrystallized from benzene-petroleum ether.

Deuterium Analyses—These were carried out by combustion of the samples to water, and reduction of the water over zinc dust to H_2 plus HD. The deuterium content of the gas was then determined by mass analysis.

Results

Influence of Fumarate on Anaerobic Deuterium Incorporation into Succinate—In contrast to the previous findings of Weinmann *et al.* (2), the present preparations, when incubated anaerobically with succinate in a medium containing D_2O, exhibited an extremely low rate of incorporation of deuterium into succinate compared to the corresponding rate of aerobic oxidation (Table I, Experiment 1 of Series I, II, and III). As indicated in Table I (Series I, Experiments 1 and 3, Series II, Experiments 1 and 4, and Series III, Experiments 1 and 4), the addition of fumarate, although

[2] This modification involved the initial elution of succinate and malate in one fraction with 90 to 100 ml. of 2.5 N formic acid. Changing to 5 N formic acid resulted in the almost immediate elution of fumarate as a distinct peak, followed by the emergence of inorganic phosphate. The 2.5 N formic acid eluates containing both succinate and malate were lyophilized, dissolved in water, neutralized to pH 8.0 to 8.5 with KOH, and again passed through Dowex 1-formate columns. Succinate and malate were then separated by elution with continually increasing formic acid concentrations as described by Busch *et al.* (8).

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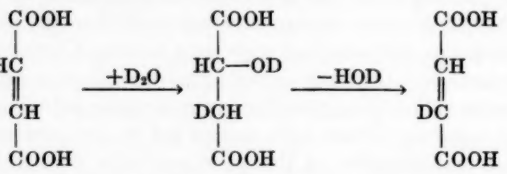
occasionally inhibiting aerobic succinate oxidation, invariably greatly stimulated the incorporation of deuterium into succinate under anaerobic conditions.

The effective stimulation of deuterium incorporation into succinate by fumarate, if interpreted in terms of a simple reversal of the classical succinic dehydrogenase reaction, could mean that the deuterium appearing in the succinate either arose directly from the medium in the course of reduction of unlabeled fumarate or arose from a reduction of previously labeled fumarate. The latter possibility was ruled out by the observations described in the following section.

Stereospecific Behavior of Fumarase—The succinic dehydrogenase preparations used throughout this study contained sufficient fumarase to establish rapidly the fumarate-malate equilibrium. Such an equilibration, if catalyzed in the non-stereospecific manner described by Equation 3, would yield labeled fumarate. Hence, the finding of deuterium in succinate and the stimulation of this deuterium incorporation by fumarate might have at least partially reflected a prior incorporation of deuterium into fumarate by a non-stereospecific behavior of fumarase.

(3)



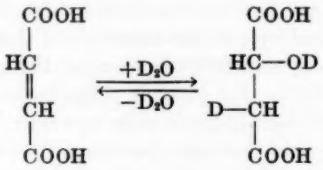
That such was not the case can be noted from an experiment in which fumarate alone was incubated with the enzyme preparation under conditions identical to those in which the exchange reaction was determined (Table I, Series II, Experiment 5). Thus, while the malate exhibited an extremely high deuterium content, as anticipated, the fumarate was exceedingly low in its content of deuterium. The reaction proceeded long after the fumarase equilibrium had been reached,[3] so that there was ample opportunity for the fumarate to become more highly labeled if the fumarase behaved in a non-stereospecific manner, according to Equation 3. Similarly, in the experiments designed to test the influence of fumarate on the extent of deuterium incorporation into succinate (Table I, Series I, Experiments 2 and 3, Series II, Experiments 2, 3, and 4), while the succinate exhibited increased deuterium incorporation with time, the deuterium content of fumarate, if significant at all, was extremely low, and the deuterium content of malate remained high and essentially constant, at close to the theoretical

[3] The half time for reaching fumarase equilibrium was approximately 2 hours with the enzyme preparation used in Series II and 4½ hours with the enzyme preparation used in Series III.

values of 0.82 and 0.86 atom of deuterium per molecule, respectively (assuming no isotope effect). These results thus tend to strengthen the belief that the deuterium incorporated anaerobically into succinate in the absence or presence of fumarate arises directly from the medium and not from labeled fumarate.

The finding that fumarase in its back-reaction behaves with strict specificity in removing the identical molecule of H_2O from *l*-malate as was incorporated into fumarate in its forward reaction, is in accordance with Equation 4, and in agreement with similar results obtained by Fisher *et al.* (4) with crystalline fumarase.

(4)



Variability of Enzyme Preparations in Extent of Deuterium Incorporation into Fumarate—The previous conclusions regarding the stereospecific behavior of fumarase and the probability that the deuterium incorporated into succinate during the anaerobic exchange reaction arises directly from the medium are based on the low deuterium values recorded for fumarate (Table I, Series I and II). In other experiments, however, a higher deuterium incorporation into fumarate was observed. Thus, when fumarate was permitted to undergo the fumarase-catalyzed equilibrium in a medium enriched with D_2O (Table I, Series III, Experiment 5), the deuterium content of the reisolated remaining fumarate was significantly increased over that of a similar experiment recorded for a different enzyme preparation (Table I, Series II, Experiment 5). Moreover, in contrast to the previously discussed experiments in Table I (Series I and II), this same preparation (Series III), as well as that used for the experiment recorded in Table I, Series IV, when incubated anaerobically with succinate in the presence of fumarate, led to even higher deuterium incorporation into fumarate. The increase in deuterium levels of succinate, with time, was accompanied by similar high increases of deuterium incorporation into fumarate and a concomitant increase of malate labeling (due to malate originating from labeled fumarate), eventually exceeding the theoretical value of 0.86 atom of deuterium per molecule for malate derived from unlabeled fumarate (Table I, Series III, Experiments 2, 3, and 4). Although the reason for the differences in behavior of the various preparations made in practically identical fashion is not clear at the present time, it should be noted that certain correlations, to be discussed later, exist between the

oxidizing activities of the various preparations and their activities with respect to deuterium incorporation into both succinate and fumarate.

Deuterium Distribution in Enzymatically Produced Deuteriosuccinate—The original postulate of Ogston (14) that citrate as an enzyme substrate complex may behave as an asymmetrical molecule was subsequently supported by the experiments of Potter and Heidelberger (15) and Lorber *et al.* (16). Recent studies on reactions involving hydrogen transfers to and from pyridine nucleotides have shown that these reactions are sterically specific with respect to the pyridine nucleotide (11, 17–20) and with respect to ethanol as well, when studied in the case of alcohol dehydrogenase (21). On the assumption that the exchange observed in the present experiments is catalyzed by a single enzyme and that the same enzyme is concerned in the aerobic oxidation of succinate, the occurrence of a similar sterically specific behavior for succinic dehydrogenase could be tested. Thus, if the exchange reaction resulted in succinate labeled specifically in two of its four methylene hydrogen positions, its oxidation by the same enzyme should yield essentially unlabeled fumarate. Such an experiment, in which deuteriosuccinate obtained by enzymatic exchange of deuterium into normal succinate in the presence of fumarate was enzymatically oxidized to fumarate, is recorded in Table II, and clearly indicates that the resulting fumarate retained (per molecule) about half the deuterium originally present in the succinate. These findings, therefore, lend themselves to the interpretation that during the course of the anaerobic exchange reaction all four methylene hydrogens of succinate became labeled. The apparent discrepancy between the deuterium content of succinate before and after 50 per cent oxidation is probably due to an isotope effect which causes preferential oxidation of the unlabeled succinate molecules (22) leading to a higher apparent deuterium content of the residual succinate and a somewhat lower than theoretical content of the resulting fumarate. It may be pointed out that identical results were observed with the deuteriosuccinate obtained from experiments in which the respective fumarate pools were unlabeled or labeled (Table II, Experiments 1 and 2, respectively).

Use of Enzymatically Formed Deuteriosuccinate As Substrate for Anaerobic Exchange Reaction—In order to duplicate more closely the experimental procedure followed by Weinmann *et al.* (2), deuteriosuccinate samples isolated from various experiments listed in Table I were incubated anaerobically with the heart particle preparations in a normal water medium with or without added fumarate. The results are outlined in Table III.

The main point to note is the relatively small decrease in the deuterium content of the deuteriosuccinate, even when incubated with fumarate. While this finding might at first inspection suggest a discrepancy with the

experiments reported in Table I, on closer examination it becomes clear that the difference is due to a difference in sensitivity of the two methods employed for the detection of exchange. This can be illustrated by a consideration of the following figures. The enzyme preparations in Experiments 2 and 3, Table III, when used for similar time periods in experiments with normal succinate and heavy water (Table I), showed about 0.6 per cent of maximal theoretical exchange without fumarate and about 9 per

TABLE II

Enzymatic Oxidation of Enzymatically Labeled Deuteriosuccinate

Acid isolated and conditions	Deuterium content					
	Experiment 1			Experiment 2		
	Dilution factors	Atom per cent excess*	Atom per molecule†	Dilution factors	Atom per cent excess*	Atom per molecule†
Deuteriosuccinate obtained from anaerobic exchange reaction	No dilution	0.502	0.0301	4.1	0.402	0.0989
Succinate after approximately 50% enzymatic oxidation of above deuteriosuccinate	2.1	0.341	0.0430	4.4	0.505	0.133
Fumarate resulting from above oxidation of deuteriosuccinate	3.5	0.098	0.0137	5.0	0.222	0.0444

Experiment 1, D-succinate from Experiment 3, Series I, Table I, oxidized with the enzyme from Series IV; Experiment 2, D-succinate from Series IV, Table I, oxidized with the enzyme from Series III.

* Experimental values.

† Values corrected for dilution and calculated on the basis that 1 atom of deuterium per molecule of succinic acid and fumaric acid would correspond to a value of 16.7 and 25.0 atoms per cent excess, respectively.

cent with fumarate. If a similar degree of exchange had occurred, starting with deuteriosuccinate in Table III, the exchange would certainly have been undetectable in the experiments without fumarate and would have been just on the border of significance with fumarate. The actual results in Experiments 1 and 3, Table III, are in agreement with this expectation, while those in Experiment 2 show somewhat higher exchange values than would have been expected from the above considerations.

Our inability to observe rapid exchange of deuterium into or out of succinate, when incubated alone anaerobically with the Keilin and Hartree heart particle preparations, is not in agreement with the rapid exchange of

hydrogen into dideuteriosuccinate originally reported by Weinmann *et al.* (2), who used a rabbit kidney preparation. The discrepancy between these findings and those of the earlier workers might perhaps be due to differences in the nature of the enzyme preparations used. Similar experiments, however, with dialyzed and undialyzed supernatant fractions and

TABLE III

Enzymatic Anaerobic Exchange of Enzymatically Labeled Deuteriosuccinate with Normal Water

Treatment of deuteriosuccinate obtained from anaerobic exchange reaction	Deuterium content of succinate								
	Experiment 1			Experiment 2			Experiment 3		
	Dilution factors	Atom per cent excess*	Atom per molecule	Dilution factors	Atom per cent excess*	Atom per molecule†	Dilution factors	Atom per cent excess*	Atom per molecule†
None	No dilution	0.502	0.0301	4.1	0.402	0.0989	4.1	0.356	0.0876
After anaerobic incubation with succinic dehydrogenase	2.3	0.209	0.0288	5.0	0.265	0.0795	2.9	0.503	0.0876
After anaerobic incubation with succinic dehydrogenase in presence of equivalent concentration of fumarate				5.4	0.220	0.0713	2.9	0.440	0.0765

Experiment 1, D-succinate from Experiment 3, Series I, Table I, incubated anaerobically for 16 hours with the enzyme preparation from Series IV; Experiment 2, D-succinate from Experiment 1, Series IV, Table I, incubated anaerobically for 10 hours with the enzyme preparation from Series III; Experiment 3, D-succinate from Experiments 2, 3, and 4, Series III (pooled), incubated anaerobically for 4 hours with the enzyme preparation from Series II.

* Experimental values.

† Values corrected for dilution and calculated on the basis that 1 atom of deuterium per molecule of succinic acid corresponds to a value of 16.7 atoms per cent excess.

washed residues from rabbit kidney homogenates prepared according to Colowick *et al.* (23, 24), invariably yielded extremely low values of anaerobic deuterium incorporation into succinate compared to the rate of aerobic succinate oxidation. It should be emphasized that, in the experiments of Weinmann *et al.* (2), extremely large quantities of enzyme were used and methylene blue reduction rather than oxygen uptake was used as the basis for comparison with the rate of anaerobic exchange. These differences in experimental conditions and procedure may account in part for the apparent discrepancies observed.

DISCUSSION

Mechanism of Anaerobic Labeling of Succinate—The results obtained in this study are most readily explained by formulation of Equations 5 and 6,

(5) $^{-}OOC—CH_2—CH_2—COO^{-} + 2X \rightarrow {}^{-}OOC—CH{=}CH - COO^{-} + 2X^{-} + 2H^{+}$

(6) $^{-}OOC—CH{=}CH—COO^{-} + 2X^{-} + 2D^{+} \rightarrow {}^{-}OOC—CHD—CHD—COO^{+} + 2X$

in which Equation 5 represents succinate as a reductant of a natural carrier X and Equation 6 represents fumarate as reoxidizing the reduced carrier X in a medium enriched with D_2O. Alternatively, the reduction of X would yield XH_2, the hydrogens of which would be rapidly exchangeable with the medium D_2O. The resulting XD_2 would subsequently be reoxidized by fumarate, yielding X and deuteriosuccinate. According to this scheme, the addition of fumarate would promote the incorporation of deuterium into succinate by establishing a new equilibrium position in which the rates of both Equations 5 and 6 would be increased.[4] The available data do not permit a definitive conclusion as to the involvement of only one enzyme in the catalysis of both Equations 5 and 6. Thus, while Equation 6 in all probability is due to the generally described succinic dehydrogenase, the possibility must be entertained that Equation 6 may be catalyzed by an enzyme similar to the fumaric reductase isolated from yeast by Fischer *et al.* (25–27). However, in such a case, the carrier X would have to be capable of interacting with both enzyme systems. Massey *et al.* (28) have recently reported evidence supporting the view that a single enzyme is responsible for Equations 5 and 6. Using a highly purified flavoprotein from beef heart, they showed that the enzyme served as both a succinic dehydrogenase and a fumarate reductase, and that the reduced enzyme could be reoxidized by fumarate.

Significance of Fumarate Labeling—According to the scheme proposed, the deuterium appearing in the succinate should arise directly from the medium and not from a reduction of labeled fumarate. The results outlined in Table I, Series I and II, which show that the fumarate remains essentially unlabeled as the deuterium incorporation into the corresponding succinate increases with time are, therefore, in agreement with the proposed mechanism. As already pointed out, the fumarase in these preparations functions stereospecifically and does not lead to labeling of fumarate. The small amount of deuterium found in the fumarate in these experiments, if indeed significant at all, probably arises from deuteriosuccinate by the non-stereospecific reversal of Equation 6 as formulated by the Equation 7.

[4] At the new equilibrium position, Equation 6 would be increased in rate simply because of the increased fumarate concentration, while Equation 5 would be increased in rate because of the increased concentration of oxidized carrier resulting from fumarate addition.

(7) $^{-}OOC—CHD—CHD—COO^{-} + 2X \rightarrow$

$$^{-}OOC—CH{=}CD—COO^{-} + 2X^{-} + D^{+} + H^{+}$$

The low values for fumarate labeling probably reflect (*a*) the low concentration of labeled succinate undergoing dehydrogenation during the experimental period and (*b*) the dilution of the small amount of isotopic fumarate thus formed by the initially added normal fumarate. It should be added that the non-stereospecific reversal of Equation 6 would be expected to lead eventually to extensive labeling of fumarate. It can be shown from a theoretical consideration of this process that, whereas the rate of labeling of succinate is directly proportional to the concentration of unlabeled succinate remaining at any time, the rate of labeling of fumarate is directly proportional to the concentration of labeled succinate (minus labeled fumarate) at any time. The theoretical time-course for labeling of fumarate would, therefore, involve a lag period. Thus, one might expect that under conditions by which succinate labeling became extensive (*e.g.* with longer incubation or with more active enzyme preparations) the degree of fumarate labeling would become larger. The high degree of fumarate labeling shown in Table I, Series III and IV, is, therefore, not in itself inconsistent with the proposed hypothesis. However, the fact that the isotope levels in the corresponding succinate were no higher than those of the experiments in Series I and II and that the rate of fumarate labeling was constant with time and thus independent of the concentration of labeled succinate, makes it difficult to reconcile the results of Series III and IV with those of Series I and II. Nevertheless, even the results summarized in Table I, Series III, are in agreement with the proposed scheme in that the bulk of the deuterium in the succinate arises directly from the medium and not secondarily from labeled fumarate, and that the extent of fumarate labeling depends on the presence of succinate. Thus, in all experiments the deuterium content per molecule of fumarate was always less than that of succinate and, by comparison of Experiments 4 and 5 (Table I, Series III), one notes that the presence of succinate increased the extent of fumarate labeling by a factor of approximately 3-fold. The appearance of deuterium in fumarate during the anaerobic incubation of normal fumarate alone with the enzyme preparation in heavy water (Table I, Series III, Experiment 5) may possibly reflect a prior net synthesis of small amounts of deuteriosuccinate attributable to traces of reducing substances in this enzyme preparation.

Although, as indicated before, both sets of data obtained in this study (*i.e.* low and high fumarate labeling accompanying succinate labeling) are compatible with the proposed scheme, the reasons for the differences in behavior of different preparations are elusive and not clear at the present time. Nonetheless, there were certain differences in the history and prop-

erties of these preparations which appear to be worth noting. Thus, the preparations made from relatively fresh tissue showed high succinoxidase activity in the presence of fumarate and were only slightly inhibited by addition of the latter. These preparations (Table I, Series I and II) exhibited high rates of deuterium incorporation into succinate with correspondingly little labeling in the fumarate. On the other hand, preparations from tissues stored in the frozen state for periods ranging from 2 to 3½ months were relatively less active with respect to succinate oxidation in the presence of fumarate and were inhibited as much as 50 per cent by the latter. These same preparations (Table I, Series III and IV) gave equal or lower rates of deuterium incorporation into succinate but much higher levels of fumarate labeling. One could possibly invoke the presence in these latter preparations of an enzyme stimulated by succinate, which in some unknown manner introduces deuterium into fumarate, in order to explain the two different sets of data obtained in this study. It should be noted, however, that such an explanation is presented only as a possible means of reconciliation between two sets of data and is completely devoid of experimental basis.

Stereochemistry of Dehydrogenation of Succinate—As pointed out in a previous section, on the assumption that the exchange reaction is catalyzed by a single enzyme and this same enzyme is concerned in the oxidation of succinate to fumarate, the data presented in Table II would indicate that during the anaerobic exchange reaction all four hydrogen positions of succinate became labeled. This random labeling of succinate has been re-

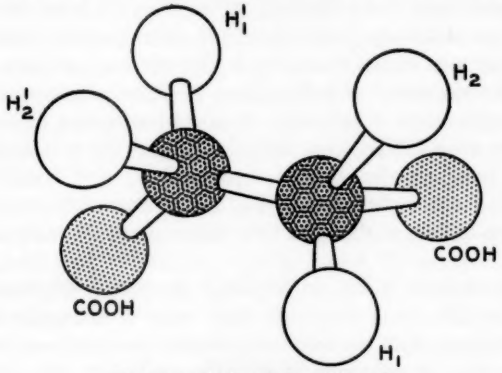


DIAGRAM 1. Succinic acid model

ferred to above as resulting from the "non-stereospecific" nature of Equations 4 to 7. In order to aid in the discussion of the stereochemistry of this process, Diagram 1 accompanying has been reproduced. In this diagram, the hydrogen atoms marked H_1 and H_1' are stereochemically indis-

tinguishable. The atoms marked H_2 and H_2' are also stereochemically indistinguishable. H_1 or H_1' is stereochemically readily distinguishable from H_2 or H_2', *e.g.* by the following test: When examined from a point outside the molecule on the axis joining the methylene carbon atoms, the H_1, H_2, and COOH groups attached to the proximal methylene carbon will always appear in that order, reading clockwise. Such a test for the asymmetry of a carbon atom bearing two like groups has been suggested previously by Schwartz and Carter (29). The Ogston (14) test of 3 point attachment may also be applied, but is in no way a unique criterion for stereochemical asymmetry.

Whenever dehydrogenation occurs by removal of a pair H_1, H_2', this is stereochemically indistinguishable from removal of the pair H_1', H_2, and there is no enzyme or other device which could make that distinction. On the other hand, when dehydrogenation occurs by removal of the pair H_1, H_1', this is stereochemically readily distinguishable from removal of the pair H_2, H_2', and an enzyme could readily make that distinction. The fact that the enzyme studied here does not distinguish between the 4 hydrogen atoms of succinic acid must mean either that (*a*) the enzyme is catalyzing the removal of a pair H_1, H_2' (= H_1', H_2), in which case there is no chance for selective action on one pair of hydrogens, or that (*b*) the enzyme is catalyzing indiscriminately the removal of either an H_1H_1' or an H_2H_2' pair, in spite of the fact that there is a theoretical possibility for selective action. In view of the fact that other enzymes exert stereospecificity with respect to methylene hydrogens in all known cases so far studied, it would appear more likely that (*a*) rather than (*b*) is operative here.

Thus far, no statement has been made regarding the configuration of the carboxyl groups of the succinate or the steric mechanism of the reaction, since the argument is independent of these considerations. If the staggered configuration is assumed, (*a*) corresponds to a trans elimination of 2 hydrogen atoms, H_1H_2' (= $H_1'H_2$), whereas (*b*) would correspond to the indiscriminate cis elimination of pairs H_1H_1' and H_2H_2'.[5] Whether (*a*) or (*b*) applies, the random labeling in the succinate would appear to justify the application of the term "non-stereospecific" to the action of the enzyme.

Quantitative Aspects of Rate of Exchange Reaction—Whereas Weinmann *et al.* (2) were able to demonstrate high rates of anaerobic exchange of succinate hydrogens with the medium in kidney preparations, and regarded the exchange rate as reflecting closely the oxidation rate, other reports, including the present one, suggest that the exchange rate is relatively small compared with the possible rate of succinate oxidation. Erlenmeyer *et al.*

[5] The authors are grateful to Dr. Harvey F. Fisher for bringing these considerations to our attention.

(3) incubated deuteriosuccinate anaerobically with a concentrated beef muscle paste, and under such conditions oxidized the succinate by the dropwise addition of methylene blue over a period of 6 hours. The fumarate formed as well as the unchanged succinate showed an approximately 20 per cent higher hydrogen to deuterium ratio than the initial succinate. The extent of exchange obtained by them, in the presence of continually formed fumarate, closely corresponds to the values obtained in the present study when succinate and fumarate were incubated together. Even with the 14- to 15-fold stimulatory effect of fumarate, the extent of deuterium incorporation into succinate does not approach the values obtained by Weinmann *et al.* (2). Nevertheless, the observed exchange rates are highly significant and readily measurable by the techniques described herein.

However, difficulties in demonstrating with certainty deuterium exchange into succinate as catalyzed by succinic dehydrogenase preparations have been experienced by Geib and Bonhoeffer (30). Although these authors mention their repeated attempts under varying conditions to demonstrate such an exchange reaction, no data are presented nor any description of the various experimental conditions employed. More recently, Swim and Krampitz (31), in attempting to ascertain the quantitative significance of the tricarboxylic acid cycle in *Escherichia coli*, studied the anaerobic oxidation of acetate in the presence of fumarate added as an oxidant. Under these conditions, experiments with acetate-1-C^{14} showed that most of the isotope was located in the succinate and that the residual fumarate as well as respiratory carbon dioxide were essentially unlabeled. Although these results may seem to contradict the present interpretation of the anaerobic exchange reaction, as based on the stimulatory effects of added fumarate, one should take into consideration the increased complexity of the electron transfer reactions which must occur during the course of acetate utilization in the *E. coli* system. It is clear that a preferential oxidation of electron donors other than succinate could explain the observed results.

The authors are indebted to Dr. Theodore Enns of The Johns Hopkins University School of Medicine, working under Veterans Administration contract No. V1001-M527, for his kind cooperation in carrying out the deuterium analyses.

SUMMARY

1. Heart particle preparations catalyze a slow anaerobic exchange of deuterium from water into succinate. The rate of this exchange reaction is extremely slow compared to the rate of theoretical aerobic oxidation and is stimulated about 15-fold by the addition of fumarate.

2. The deuterium appearing in the succinate arises directly from the medium and not secondarily from labeled fumarate.

3. Equilibration of fumarate with malate in D_2O by fumarase does not lead to appreciable fumarate labeling, thus indicating a stereospecific behavior of fumarase, in agreement with the conclusions of Fisher *et al.*

4. Deuteriosuccinate obtained by the anaerobic enzymatic exchange reaction, when oxidized aerobically by the same enzyme preparation, yields fumarate which retains half the isotope content (as atoms D per molecule) found in the original succinate. These results are discussed in terms of the stereochemistry of the succinic dehydrogenase reaction, and it is suggested that the term "non-stereospecific" be applied to the action of the enzyme in this case.

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THIOLESTERASE ACTIVITY OF PAPAIN*

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Recently it has been clearly shown that certain of the proteolytic enzymes, especially those of the gastrointestinal tract, are capable of catalyzing the hydrolysis of the carboxyl carbon-oxygen bond of acylamino acid esters as well as the carboxyl carbon-nitrogen bond of peptides and acylamino acid amides (2–5). The only indication that proteolytic enzymes may catalyze the hydrolysis of the analogous carboxyl carbon-sulfur bond of acylamino acid thiol esters has been the report of Goldenberg *et al.* that the odor of ethyl mercaptan can be detected when acetylphenyl-DL-alanine thiol ethyl ester is incubated with chymotrypsin (6). Strecker *et al.* have demonstrated that extracts of ox brain will catalyze the hydrolysis of the thiol ester linkage of *S*-alanylglutathione (7). In this case it has not been established whether the thiolesterase of ox brain is specific for the thiol ester linkage or can also split the corresponding oxygen esters and amides. The thiolesterase activity of the plant proteolytic enzymes such as papain has not been investigated. Previous experiments have shown that benzoylglycinamide is an active synthetic substrate for papain (8). The experiments reported here demonstrate that papain also catalyzes the hydrolysis of the thiol ester linkage of benzoylglycine thiol ethyl ester (BGTEE) and benzoylglycine thiol isopropyl ester (BGTIPE) and the oxygen ester linkage of benzoylglycine ethyl ester (BGEE).

EXPERIMENTAL

Materials

Papain—The crystalline papain employed in these experiments was crystalline mercuripapain kindly supplied by Dr. Emil Smith (9). In the experiments in which there is no indication that crystalline papain was used, the papain employed was prepared according to Grassmann (10) from crude dried papaya latex obtained from the Wallerstein Laboratories.

Benzoylglycinamide—This compound was prepared by the mixed anhydride method of Boissonnas (11). 18 gm. of hippuric acid were suspended in 120 ml. of dry methylene chloride and cooled to −10°. 14.2 ml. of cold dry triethylamine were added, and 9.6 ml. of ethyl chlorocarbonate, and the reaction mixture allowed to stand at −10° for 10 minutes. Am-

* An abstract of a preliminary report of this work has been published (1).

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monia gas was passed into the solution of the anhydride. After standing for 3 hours at room temperature, the solvent and excess ammonia were removed under a vacuum and the residue was recrystallized twice from water. Yield 12.8 gm., m.p., 185–186°.

$C_9H_{10}O_2N_2$ (178.2). Calculated, N 15.72; found, N 15.83, 15.77

Benzoylglycine Ethyl Ester—28 gm. of glycine ethyl ester hydrochloride were suspended in 200 ml. of methylene chloride. The suspension was cooled to −5°. 50.6 gm. of triethylamine were added. While stirring, 31 gm. of benzoyl chloride were added dropwise. After all of the benzoyl chloride had been added, the solution was allowed to come to room temperature, at which the reaction mixture was stirred for 30 minutes, and the triethylamine hydrochloride which precipitated was filtered and washed with methylene chloride. After removal of the methylene chloride from the combined filtrate and washings by vacuum distillation, the product crystallized on standing. The benzoylglycine ethyl ester was recrystallized from methanol-water and from ethyl acetate-petroleum ether. Yield, 39 gm., m.p., 59–60°.

$C_{11}H_{13}O_3N$ (207.2). Calculated, N 6.76; found, N 6.70, 6.76, 6.78

Benzoylglycine Thiol Ethyl Ester—This compound was prepared essentially by the method employed by Jeger *et al.* (12) to prepare the corresponding methyl thiol ester. The acid chloride prepared from 40 gm. of benzoylglycine was divided into four equal portions and added to four pressure flasks which contained 50 ml. of anhydrous benzene cooled to −5°. 13 ml. of practical ethyl mercaptan and 6.1 ml. of pyridine were added to each pressure flask. Each flask was stoppered and allowed to come to room temperature. After standing for 24 hours at room temperature, the contents of each of the flasks were poured into a separatory funnel with the aid of 50 ml. of water and 50 ml. of ether. All the flasks were then washed with 50 ml. of ether and 50 ml. of water. The washings were added to the separatory funnel. The ether phase was removed and the aqueous phase extracted twice with 300 ml. of ether. The combined ether extracts were washed with cold saturated $KHCO_3$ and cold water and dried over anhydrous sodium sulfate. On concentration of the ether solution in vacuum, crystallization occurred. Recrystallization from water gave a product which melted at 85–86°.

$C_{11}H_{13}O_2NS$ (223.3). Calculated, N 6.27; found, N 6.32, 6.24

Benzoylglycine Thiol Isopropyl Ester—This compound was prepared with similar yields by the method described for the synthesis of thiol ethyl ester, except that 14.2 ml. of isopropyl mercaptan were substituted for the ethyl

mercaptan. On recrystallization from methanol-water, the product melted at 80–81°.

$C_{12}H_{15}O_2NS$ (237.3). Calculated, N 5.90; found, N 5.99, 6.08

Analytical Methods

The rate of hydrolysis of the amide was followed by the Conway microdiffusion technique previously described (13). In order to determine the rates of hydrolysis of the thiol esters, they were converted into the colored iron-hydroxamic acid complex by the procedure employed by Lipmann and Tuttle for the determination of acetyl phosphate (14). The colorimetric measurements were carried out with a Klett colorimeter in a total volume of 6 ml., or in a Bausch and Lomb colorimeter in 13 mm. colorimeter tubes with a 550 mμ monochromatic filter and a total volume of 3 ml. The standard curves for each of the thiol esters are linear up to 10 mμ per 6 ml. The standard curves of BGTEE and BGTIPE possessed identical slopes which, for the Klett colorimeter, were 50 scale units per micromole and for the Bausch and Lomb instrument 0.135 optical density per micromole. The hydroxamic acids of the acylated amino acids are quite unstable in strong acid solution and consequently the color developed fades rapidly. This is indicated by Fig. 1. The optical density must be determined within 3 or 4 minutes after acidification in order to obtain reliable values. The hydroxamic acids of the acylamino acids are stable at pH 5.4 up to 24 hours.

The rate of hydrolysis of BGEE was followed by a modification of the method of Hestrin (15). In this method the ester is converted to the hydroxamic acid, which is determined as a colored iron-hydroxamic acid complex. The final volume of the solution analyzed was 3 ml. with the Bausch and Lomb colorimeter and 6 ml. with the Klett. The acylated amino acid-hydroxamic acids are unstable in alkaline solutions, so that after 6 hours appreciable loss of hydroxamic acid has occurred. In order to minimize this loss, one-sixth of the final volume of the solution containing 12 per cent trichloroacetic acid in 1.67 N HCl was added to the alkaline solution 10 minutes after the sample was added. This reduces the pH to about 5.4, at which pH the hydroxamic acid is stable up to 24 hours. Under these conditions the standard curve for BGEE has a slope of 0.100 optical density per micromole when the Bausch and Lomb colorimeter is employed.

Results

Since the hippuryl derivatives have been shown to be specific substrates for a number of proteolytic enzymes (8), the thiol ethyl and the thiol isopropyl esters of benzoylglycine were prepared, and a study of the papain-catalyzed hydrolysis of these specific substrates was undertaken.

The rate of papain-catalyzed hydrolysis of BGTEE is shown in Fig. 2.

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Papain activated by cysteine or cyanide will catalyze the hydrolysis of the BGTEE. In the absence of enzyme and activator, no significant hydrolysis of the amino acid thiol ester occurs in 2 hours in 40 per cent methanol between pH 5.0 and 7.5. However, in the presence of an activator hydrolysis occurs when the enzyme is absent, as indicated by the lower curves in

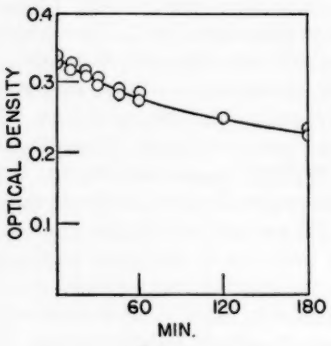

FIG. 1

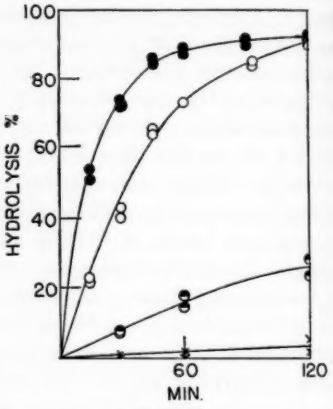

FIG. 2

FIG. 1. Stability of the benzoylglycine hydroxamic acid-iron complex in acid solution. 2.5 μmoles of benzoylglycine thiol ethyl ester in 0.5 ml. were added to 0.5 ml. of neutralized 2 M hydroxylamine and 0.5 ml. of 0.1 M acetate buffer, pH 5.4. After 10 minutes, 0.5 ml. of 12 per cent trichloroacetic acid, 0.5 ml. of 4 N HCl, and 0.5 ml. of 5 per cent $FeCl_3$ in 0.1 N HCl were added. The optical density of the solution was then determined at the times noted.

FIG. 2. Hydrolysis of benzoylglycine thiol ethyl ester catalyzed by crystalline papain. ●, cysteine-activated papain; ○, cyanide-activated papain; ◓, cysteine, no enzyme; ×, cyanide, no enzyme. The final concentrations of the components of the incubation media were BGTEE 0.025 M, cysteine 0.025 M or cyanide 0.05 M, Versene 0.002 M, citrate buffer, pH 5.0, 0.05 M, papain 0.03 mg. of protein N per ml., methanol 35 per cent; temperature, 37°.

Fig. 2. This non-enzymatic hydrolysis is related to the nature of the activator since the effect is greater with cysteine than with cyanide. Methanol is added to the components of the reaction media to increase the solubility of the substrate.

In a similar manner papain catalyzes the hydrolysis of benzoylglycine thiol isopropyl ester, as indicated in Table I. At various levels of enzyme concentration the rates of hydrolysis for the two thiol esters are essentially the same. The specificity of the enzyme is such that it does not differentiate between the isopropyl and the ethyl group of the two thiol esters and,

consequently, only the benzoylglycine thiol ethyl ester was employed on the subsequent studies. The amount of thiol ester hydrolyzed is a direct function of the concentration of the enzyme present within the range of enzyme concentrations studied. This indicates that the hydrolysis of the thiol ester is a true catalytic property of the papain. Furthermore, the enzymatic hydrolysis of the thiol ester does not occur when the papain is inactivated by heating for 5 minutes at 100°.

The effect of variation of pH between pH 5.0 and 7.5 on the enzymatic hydrolysis has been studied, and the results of these experiments, given in Fig. 3, indicate that the cleavage of the thiol ester bond by the papain is quite insensitive to changes in hydrogen ion concentration throughout the pH range studied. There is a slight increase in the total hydrolysis of the

TABLE I

Relative Rates of Hydrolysis of Benzoylglycine Thiol Ethyl Ester and Benzoylglycine Thiol Isopropyl Ester by Papain

The final concentrations of the components of the incubation media were thiol ester, 0.025 M; cysteine, 0.025 M; citrate buffer, pH 5.0, 0.04 M; methanol, 30 per cent; temperature, 37°.

Enzyme concentration	Benzoylglycine thiol ethyl ester hydrolyzed	Benzoylglycine thiol isopropyl ester hydrolyzed
mg. N per ml.	*μmoles per ml. per hr.*	*μmoles per ml. per hr.*
0.1	20.5	25.0
0.02	11.5	12.0
0.01	9.5	9.5
0.00	5.0	6.5

thiol ester at pH 7.0 and 7.5, but this increase is due to the increase in the non-enzymatic hydrolysis at the higher pH values. A similar broad pH optimum has been observed by Kimmel and Smith in the papain-catalyzed hydrolysis of benzoylargininamide (9). The observation that the hydrolysis of the thiol ester and the amide show similar pH effects suggests that the catalysis of the two distinct substrates by the enzyme may follow the same general mechanism.

The basic requirements for the enzymatic hydrolysis of the thiol ester at pH 5.0 are shown in Fig. 4. Kimmel and Smith reported that crystalline mercuripapain required both Versene and an activator such as cyanide or cysteine to catalyze the hydrolysis of benzoylargininamide. As indicated in Fig. 4, the hydrolysis of the thiol esters requires neither activator nor Versene for maximal enzymatic activity.

In the case of the hydrolysis of the thiol esters at pH 5.0, the enzyme appears to be activated by either the mercaptan, which is formed as a product

of the hydrolysis, or by the direct action of the thiol ester on the enzyme. Citrate has a marked stimulatory effect upon the thiolesterase activity of papain.

The requirements and effect of the activators at pH 7.5 are considerably different from those observed at pH 5.0. At pH 5.0, an activator is not

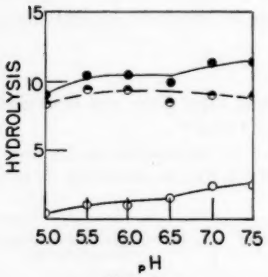

FIG. 3

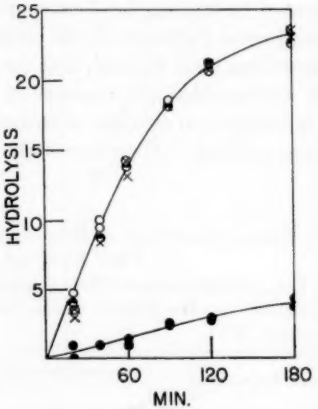

FIG. 4

FIG. 3. Effect of pH on the hydrolysis of benzoylglycine thiol ethyl ester by papain. ●, complete system; ○, no enzyme; ◓, corrected for non-enzymatic hydrolysis. The final concentrations of the components of the incubation media were BGTEE 0.025 M, HCN 0.05 M, citrate-phosphate buffer, 0.05 M, papain 0.057 mg. of protein N per ml., methanol 30 per cent; temperature, 37°. The hydrolysis is expressed in micromoles per ml. per 30 minutes.

FIG. 4. Requirements for the hydrolysis of benzoylglycine thiol ethyl ester by crystalline papain. ○, complete system; ◓, no cyanide; ×, no Versene; and ●, acetate buffer, replacing the citrate buffer. The final concentrations of the components of the complete incubation media were BGTEE 0.025 M, cyanide 0.05 M, Versene 0.002 M, citrate buffer, pH 5.0, 0.05 M, papain 0.015 mg. of protein N per ml., and methanol 35 per cent; temperature, 37°. Hydrolysis is expressed in micromoles per ml. of incubation solution.

required for optimal thiolesterase activity of papain. At pH 7.5 cyanide shows a definite activating effect on the enzyme, as is seen in Fig. 5. This might be expected, since it is known that the activation of papain requires higher concentrations of the activator at pH 7.5 than at pH 5.0 (13). It is interesting that cysteine, which is commonly employed as an activator for papain, cannot be employed conveniently at pH 7.5 to activate the enzyme when a thiol ester is the substrate, since at this pH value there is a rapid non-enzymatic reaction between the thiol ester and the cysteine which results in the disappearance of hydroxamic acid-forming substances.

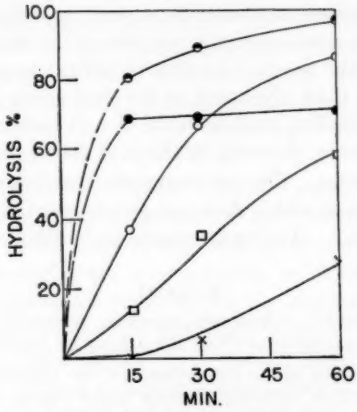

FIG. 5. Effect of activators on the hydrolysis of benzoylglycine thiol ethyl ester by papain at pH 7.5. ◓, complete system cysteine activation; ●, no enzyme cysteine activation; ○, complete system cyanide activation; ×, no enzyme cyanide activation; □, no activator. The final concentrations of the components of the incubation media were BGTEE 0.025 M, phosphate-citrate buffer, pH 7.5, 0.05 M, papain 0.06 mg. of protein N per ml., and methanol 30 per cent; temperature, 37°.

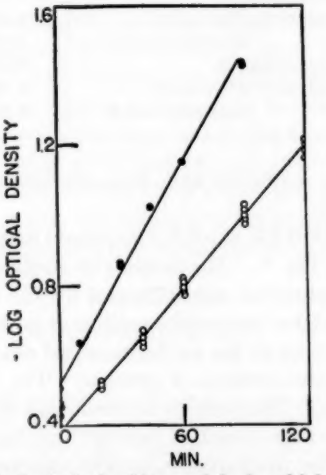

FIG. 6. Kinetics of the hydrolysis of benzoylglycine thiol ethyl ester catalyzed by crystalline papain. ●, cysteine activation; ○, cyanide activation. The final concentrations of the components of the incubation media were BGTEE 0.025 M, cysteine 0.025 M or cyanide 0.05 M, Versene 0.002 M, citrate buffer, pH 5.0, 0.005 M, papain 0.015 mg. of protein N per ml., and methanol 35 per cent; temperature, 37°.

This reaction is complete in about 15 minutes, as indicated by the broken lines in Fig. 5. The non-enzymatic reaction of the thiol ester and the cysteine appears to be the result of a sulfur to sulfur transfer of the acylamino acid group from the thiol ethyl ester to the thiol group of cysteine. Under neutral or slightly alkaline conditions the *S*-acyl cysteine which forms may undergo rearrangement, resulting in the transfer of the acyl group to the nitrogen of the cysteine. The non-enzymatic reaction results in the formation of *N*-acyl cysteine which does not give a hydroxamic acid under the conditions of analysis. Analogous reactions of thiol esters with cysteine

TABLE II

Relative Rates of Hydrolysis of Benzoylglycinamide, Benzoylglycine Ethyl Ester, and Benzoylglycine Thiol Ethyl Ester by Papain

The final concentrations of the components of the incubation media were substrate 0.025 M, HCN 0.05 M, citrate-phosphate buffer 0.05 M, methanol 30 per cent. Incubation for 1 hour at 37°.

		Protein concentration	Hydrolysis	
			Total	Per mg. protein N
		mg. N per ml.	*μmoles*	*μmoles*
At pH 5.0	Benzoylglycinamide	0.82	10.2	12.4
	Benzoylglycine ethyl ester	0.205	8.0	39.0
	" thiol ethyl ester	0.057	16.0	280.0
At pH 7.5	Benzoylglycinamide	0.82	10.7	13.5
	Benzoylglycine ethyl ester	0.205	11.0	54.0
	" thiol ethyl ester	0.057	16.0	280.0

in neutral or alkaline conditions have been described by Wieland *et al.* (16) and Strecker *et al.* (6).

The hydrolysis of BGTEE at pH 5.0 by papain follows first order kinetics. This is illustrated in Fig. 6. The proteolytic coefficient (17) at pH 5.0 for cysteine-activated hydrolysis was 0.72, and for the cyanide-activated hydrolysis 0.46. The higher proteolytic coefficient in the case of the cysteine-activated hydrolysis may be due to the increased non-enzymatic hydrolysis of the thiol ester in the presence of cysteine. The thiol esters are active substrates for papain. The proteolytic coefficient of the most active substrates for crystalline papain such as benzoyl argininamide is about 1.2 (9).

A comparison of the thiolesterase, oxygen esterase, and amidase activities of the enzyme was made by observing the rates of papain-catalyzed hydrolysis of BGTEE, BGEE, and BGA under identical conditions. The rates of hydrolysis at pH 5.0 and 7.5 are shown in Table II. At both pH values studied the rate of enzymatic hydrolysis of the oxygen ester is about

three times faster than that of the amide. The thiol ester is hydrolyzed by the enzyme about seven times faster than the corresponding oxygen ester or about twenty-one times faster than the amide. This is the first report that benzoylglycine ethyl ester can be hydrolyzed by papain.

DISCUSSION

The results reported here establish that thiol ester derivatives of amino acids are rapidly hydrolyzed by the intracellular proteinase, papain. Since the chemistry and metabolism of the thiol esters of amino acids have not been extensively investigated, it is too early to decide whether the thiol ester may be a physiological substrate for the enzyme or whether the hydrolysis of the thiol ester is a non-physiological extension of the specificity of the enzyme. It is possible that thiol esters of amino acids may be a biologically important class of substances, since these compounds possess an activated carboxyl group and could conceivably have a physiological function in the biological formation of peptide bonds (7). Although at the present time no such specific metabolic rôle can be assigned to the thiol esters of amino acids, the observations reported here certainly suggest that the thiol esters of amino acids may have some specific function.

SUMMARY

Papain exhibits a powerful thiolesterase action of the thiol ethyl and thiol isopropyl esters of hippuric acid. The thiol esters are hydrolyzed about seven times faster than the oxygen ester and about twenty-one times faster than the amide. The enzymatic hydrolysis of the thiol ester by papain is relatively insensitive to pH between pH 5.0 and 7.5. At pH 5.0 an activator is not required, but at pH 7.5 an activator must be added for optimal enzymatic action. At pH 7.5 there is a non-enzymatic reaction between the thiol ester and cysteine. In addition to the thiol esters, benzoylgycine ethyl ester is also reported as a new substrate for papain.

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THE REDUCTION OF METHEMOGLOBIN BY PHENYLHYDRAZINE UNDER ANAEROBIC CONDITIONS*

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Phenylhydrazine and oxyhemoglobin react to form methemoglobin, nitrogen, benzene, and a green pigment (1). Subsequently, any excess phenylhydrazine reduces methemoglobin to hemoglobin. The amount of methemoglobin present at the end of the complex reactions of phenylhydrazine with oxyhemoglobin therefore depends on the relative concentrations of the two reactants, the relative velocities of the reaction which forms methemoglobin, and the reaction which reduces it.

This study is concerned with the reduction of methemoglobin by phenylhydrazine under anaerobic conditions. Data are presented on the relation between methemoglobin reduced and nitrogen and benzene formed and on the apparent velocity of the reduction.

EXPERIMENTAL

Anaerobic Conditions—Because the reaction of phenylhydrazine and oxyhemoglobin results in the formation of N_2 and benzene, it is necessary to exclude oxygen rigidly in order to study the reduction of methemoglobin by phenylhydrazine. This was accomplished in the experiments reported here by using high purity nitrogen[1] and chromous sulfate (2). Chromous sulfate was prepared by reducing chromic sulfate in a Jones reductor. Warburg flasks in which the reaction was to be carried out were flushed with N_2 which had been passed through chromous sulfate. Then 0.2 ml. of 1 N chromous sulfate solution was placed in one side arm and the flask was again flushed with N_2. The CO used to measure the reduction of methemoglobin was passed through mineral oil to remove organic impurities absorbing in the ultraviolet; a KOH solution and a chromous sulfate solution were employed to remove CO_2 and O_2, respectively. Flasks thus gassed were shaken for 15 minutes before the control period started in order that the last traces of O_2 could be removed by the chromous sulfate.

Preparations of Methemoglobin Solutions—Methemoglobin was prepared

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[1] Obtained from the Linde Air Products Company.

from avian or bovine blood by treating the thoroughly washed red cells with 0.0046 M $NaNO_2$ for 30 minutes. The cells were centrifuged, washed four times with saline, laked in distilled water, and centrifuged, and the supernatant fluid was brought to a convenient methemoglobin concentration. The methemoglobin concentration was estimated by (1) spectrographic analysis with the use of extinction coefficients or, more simply, (2) by reducing the methemoglobin with dithionite in the presence of CO in Warburg vessels. Runs on aliquots of the same sample showed the results of the methods to be in good agreement. The methemoglobin solutions were quite stable for several days when stored at 4°.

Phenylhydrazine Hydrochloride—Phenylhydrazine hydrochloride was recrystallized from alcohol. Nitrogen content was determined by applying the Van Slyke manometric technique to the method of Chattaway (3), wherein phenylhydrazine is oxidized by chromic acid under vacuum in the extraction chamber of the Van Slyke apparatus and the evolved N_2 is measured. The evolved N_2 agreed, within experimental limits, with the theoretical values. The recrystallized compound remained quite stable for months when stored in air-tight brown glass bottles.

A few minutes before use, solutions of phenylhydrazine were made up and adjusted to pH 7.2 with NaOH. If gassed with N_2 and stored at 4°, such solutions are stable for several hours. The nitrogen content of the phenylhydrazine used in each run was determined by oxidation with chromic acid under anaerobic conditions in the Warburg apparatus. The evolved N_2 was measured in the usual manner. The results from this procedure were in good agreement with those of the Chattaway method (3).

Measurement of N_2 Evolved in Reduction of Methemoglobin by Phenylhydrazine—In the reduction of methemoglobin by phenylhydrazine, the measurement of evolved N_2 was carried out under rigidly anaerobic conditions in double side arm Warburg vessels containing only N_2 in the gas phase. The change in volume with time was calculated from the manometric readings. 20 per cent KOH was present in the center wells.

Measurement of Methemoglobin Reduction—The measurement of reduction was carried out under anaerobic conditions in Warburg flasks containing CO or N_2. At zero time, a known amount of phenylhydrazine was tipped into a known amount of methemoglobin in companion flasks, one of which contained CO and the other N_2 in the gas phase. Since, in the presence of CO, two gas exchanges took place simultaneously, the amount of methemoglobin reduced was calculated by subtracting the microliter change in the N_2 flask from the microliter change in the CO flask. The latter was corrected for the uptake of CO by the KOH in the center well (4).

Benzene Estimation—Spectrographic analysis for benzene was performed with the model DU Beckman spectrophotometer equipped with a cell compartment assembly for 100 mm. cylindrical cells with silica windows.

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Two cells having the lower one-third of the end surfaces masked with black tape were flushed for 10 minutes with high purity N_2 bubbled through a 1 N solution of chromous sulfate. 3 ml. of a thoroughly deoxygenated methemoglobin solution containing a total of 3.3 μmoles of methemoglobin were added to each cylinder and the gas spaces again were flushed with nitrogen. The cylinders were fitted with glass stoppers with side arm pockets so that the gas space and the side arm space were continuous. The side arms contained chromous sulfate. The thoroughly flushed apparatus was allowed to stand for 30 minutes so that the chromous sulfate would remove the last traces of O_2. At this time, 0.2 ml. of a 0.06 M solution of phenylhydrazine which had been thoroughly flushed with nitrogen was added beneath the surface of the methemoglobin solution in one of the cylinders by means of a long needle passed through a vent in the stopper. The other cylinder served as a control.

The absorption in the ultraviolet region by the vapor in the gas phase of the experimental cylinder was matched against that of the control, and the optical density change at 253 mμ was followed during the course of the reaction. At the end of the experimental period, benzene was identified by the typical absorption maxima and minima between 240 and 261 mμ.

Determination of Residual Phenylhydrazine—The Lowry *et al.* modification (5) of the Folin-Ciocalteu test for phenol was found to be an excellent method for quantitative determination of phenylhydrazine, provided that no phenol was present. The test was used to determine whether phenol was formed in the methemoglobin-phenylhydrazine reaction.

The contents of a Warburg vessel in which the reduction had been carried out under anaerobic conditions were removed by pipette and added to 10 per cent trichloroacetic acid which had been previously flushed with nitrogen. After centrifugation, 0.4 ml. of the supernatant fluid was used to develop the color. Optical density was read in matched tubes on the Klett-Summerson colorimeter, and the concentration of the reducing substance present was calculated from a standard curve.

RESULTS AND DISCUSSION

Relation between N_2 Evolved and Methemoglobin Reduced—When methemoglobin reduced by phenylhydrazine is measured by the apparent CO uptake and the N_2 evolved in companion flasks, the calculated total CO uptake is equivalent to the amount of methemoglobin reduced in a given time. The N_2 evolved also bears a stoichiometric relation to the amount of methemoglobin reduced. Fig. 1 shows the comparative time-courses of the evolution of N_2 and uptake of CO in an experiment which was not allowed to proceed to completion. At first, the evolution of N_2 increased more sharply than the uptake of CO, but eventually CO uptake became twice the volume of the N_2 evolved.

Data which bear on the relation between N_2 evolution and CO uptake under conditions of differing concentrations of phenylhydrazine and met-

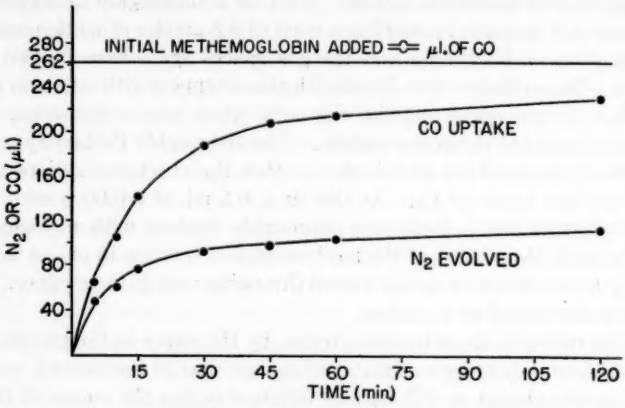

FIG. 1. Gas changes occurring during reduction of methemoglobin by phenylhydrazine in the presence of CO or N_2. The total methemoglobin present was 2.92 µmoles. 7 µmoles of phenylhydrazine were tipped in. The reaction did not go to completion.

TABLE I

Relation between N_2 Evolved and CO Uptake during Methemoglobin Reduction by Phenylhydrazine

Incubation period, 2 hours.

Experiment No.	Methemoglobin added*	Phenylhydrazine added†	N_2 evolved	CO uptake
	µl.	µl.	µl.	µl.
1	262	157	103	214
2	262	91	83	160
3	91	154	42	93
4	91	40	46	91
5	76	77	34	67
6	76	154	39	74

In Experiments 1, 2, and 5 insufficient time was allowed for the reactions to go to completion.

* CO capacity in microliters equivalent to the methemoglobin added.

† Total amount of phenylhydrazine nitrogen in microliters added.

hemoglobin are presented in Table I. In all cases, the volume of CO taken up was approximately twice that of the N_2 evolved. When the initial amount of methemoglobin, expressed as its equivalent CO capacity in microliters, exceeded that of phenylhydrazine (Line 2, Table I), the N_2 evolved

was nearly equivalent to the total amount of phenylhydrazine nitrogen present initially, although the reaction did not go to completion. Phenylhydrazine concentration equal to or greater than twice the molar concentration of methemoglobin resulted in complete reduction of the methemoglobin, if sufficient time was allowed for completion of the reaction (Lines 3, 4, and 6, Table I).

Since the CO uptake was twice the volume of the N_2 evolved and all of the phenylhydrazine nitrogen was evolved when the methemoglobin concentration exceeded that of the phenylhydrazine, the stoichiometry of the reactions in the presence of CO can be expressed as follows:

$$Hb_4^{+++} + 4OH^- + 2C_6H_5NHNH_2 \rightarrow 4H_2O + Hb_4^{++} + 2C_6H_6 + 2N_2$$

$$Hb_4^{++} + 4CO \rightarrow Hb_4^{++}(CO)_4$$

1 mole of methemoglobin is reduced while 2 moles of phenylhydrazine are oxidized. In the presence of CO, 2 volumes of CO are taken up for each volume of N_2 evolved.

Identification of Benzene—A typical benzene ultraviolet absorption spectrum was obtained when light was passed through the vapor above the methemoglobin-phenylhydrazine reaction medium. The optical density at 253 mμ increased to a maximum with time (Fig. 2). If the concentration of methemoglobin was varied while the phenylhydrazine concentration remained constant, the maximal optical density was proportional to the total amount of methemoglobin present. When CO was added to the nitrogen in the gas phase of the 100 mm. cylindrical spectrophotometer cells, it was evident from the color of the solution that most of the methemoglobin had been reduced by the phenylhydrazine. To exclude the possibility that some residual oxyhemoglobin remained after the hemoglobin solution was gassed with N_2, purified CO was added to the gas phase. In the presence of CO, the curve for the optical density change at 253 mμ remained the same as that obtained in the presence of N_2 alone. To show the effect of O_2, an experiment was conducted in which the concentration of phenylhydrazine was doubled and 2 ml. of O_2 were admitted at the time the maximal density at 253 mμ was obtained under anaerobic conditions (no CO present). A curve representing the results of this experiment superimposed (Curve III, Fig. 2) on the anaerobic curve indicates an immediate increase in optical density at 253 mμ following the admission of O_2. This marked increase was undoubtedly caused by the reaction of the residual phenylhydrazine with oxyhemoglobin formed by the oxygenation of the reduced hemoglobin. It has also been demonstrated by Beaven and White (1) that benzene is formed when phenylhydrazine reacts with oxyhemoglobin.

Chemical Estimation of Phenylhydrazine Oxidized—The Lowry *et al.* modification (5) of the Folin-Ciocalteu test for phenol was used to correlate

the phenylhydrazine oxidized with the N_2 evolved during the reduction of methemoglobin. Since the test is positive for both phenol and phenylhydrazine, it can be used to demonstrate that no phenol is formed during the reduction. Table II shows the comparison between the evolved N_2, expressed as percentage of the total phenylhydrazine nitrogen, and the percentage of the phenylhydrazine oxidized, based on the "phenol test." Thus when 7 μmoles of phenylhydrazine were allowed to react with 3.3 μmoles of oxyhemoglobin, little phenylhydrazine remained. Likewise,

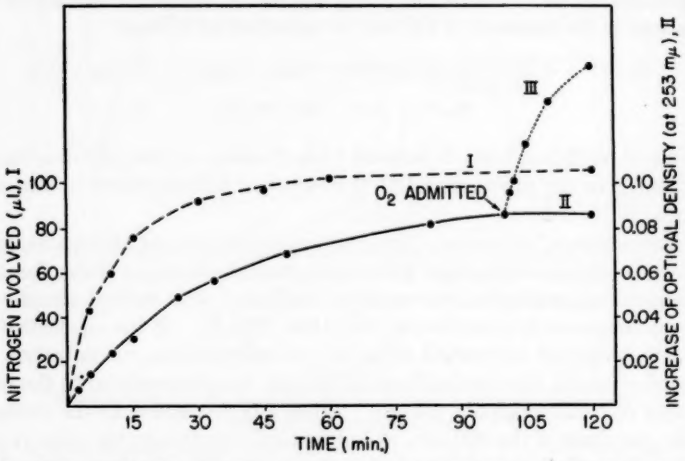

FIG. 2. Curve I indicates the rate of the reaction as measured by N_2 evolution. The increase in optical density at 253 mμ for the vapor above the methemoglobin-phenylhydrazine reaction medium when under strict anaerobic conditions (Curve II). O_2 admitted to a similar cylinder in the absence of CO at 100 minutes (Curve III).

when the amount of methemoglobin exceeded the amount of phenylhydrazine, the optical density was only slightly above that of the control. This not only indicates that practically all of the phenylhydrazine was destroyed but also that no phenol in addition to benzene was formed. Had phenol been the end-product, the optical density would have been as great as that obtained for the original unoxidized phenylhydrazine. There was usually fair agreement between the phenylhydrazine oxidized, when calculated from the N_2 evolved, and that shown to have disappeared by the use of the "phenol" test, especially when the 10 per cent trichloroacetic acid was bubbled with N_2 before the reduced hemoglobin-phenylhydrazine solution was added. Loss of some phenylhydrazine did occur, however, incident to the formation of oxyhemoglobin during the transfer of the re-

duced hemoglobin solution from the flask to the trichloroacetic acid. This caused the analytical values for residual phenylhydrazine to fall below the values calculated from the N_2 evolved.

Velocity of Reduction of Methemoglobin by Phenylhydrazine—The velocity was calculated from data for the N_2 evolved and CO uptake. Fig. 1 is a

TABLE II

Relation between N_2 Evolved and Phenylhydrazine Oxidized, Expressed As Percentage of Total Initially Present. Molar Equivalence of Methemoglobin Reduced and Phenylhydrazine Oxidized

7 μmoles of phenylhydrazine were added to varied amounts of methemoglobin.

Phenylhydrazine oxidized			Methemoglobin reduced
Calculated from N_2 evolved		By analysis*	
per cent	*μmoles*	*per cent*	*μmoles*
56	3.92	62	1.92
25	1.75	36	0.87
14	0.98	15	0.52
83	5.82	93	2.98

* By the colorimetric method of Folin and Ciocalteu as modified by Lowry *et al.* (5).

TABLE III

Velocity of Methemoglobin Reduction by Phenylhydrazine

The concentration of phenylhydrazine was 2.3 μmoles per ml. The methemoglobin concentration was 0.95 μmole per ml. (or 3.8 μeq. of Fe^{+++}).

Time	Methemoglobin reduced		k_2
min.	*per cent*	*μmole ml.*$^{-1}$	*ml. μmole*$^{-1}$ *min.*$^{-1}$
5	24.0	0.228	0.010
10	39.8	0.378	0.010
15	53.4	0.509	0.009
30	71.7	0.681	0.008

plot of the time-course of this gas exchange. There was an initial lag in the CO uptake, but eventually the CO uptake equaled twice the volume of the N_2 evolved. Second order kinetics do not exactly describe either the N_2 evolution or the CO uptake. However, an average of the two gas changes gives data which are in fair agreement with the kinetics of a bimolecular reaction. The average velocity constant (k_2) is 0.0093 ml. μmole^{-1} min.$^{-1}$. In Table III, the k_2 constants and average amount (micromoles) of methemoglobin reduced are shown for the first 30 minutes of

the reduction. Approximately 24 per cent of the methemoglobin was reduced in 5 minutes when the concentrations of the two reactants were nearly equivalent. In another experiment in which 2 equivalents of phenylhydrazine for each equivalent of methemoglobin were present, about 88 per cent of the methemoglobin was reduced in 5 minutes.

These data indicate that the velocity of the reduction of methemoglobin is high and methemoglobin formed by an initial reaction of phenylhydrazine with oxyhemoglobin would probably be reduced by any residual phenylhydrazine.

The reaction of phenylhydrazine with methemoglobin in the absence of oxygen resulted in the reduction of methemoglobin to hemoglobin and the oxidation of the phenylhydrazine to benzene and nitrogen. When the molar concentration of phenylhydrazine present initially was less than twice that of methemoglobin, all of the phenylhydrazine was decomposed. The ratio of N_2 evolved to the CO uptake was 0.5. Therefore, there appears to be little possibility that this reaction produces molecules other than benzene and nitrogen from the phenylhydrazine.

In the reaction of phenylhydrazine with oxyhemoglobin, nitrogen is evolved. The amount evolved was reported by Nizet (6) to be equivalent to the amount of oxyhemoglobin oxygen. These data indicate that the amount of N_2 depends on the quantity of methemoglobin formed, the amount of phenylhydrazine unoxidized, and the time allowed for the reduction of the methemoglobin by the residual phenylhydrazine, since the latter reaction also results in the evolution of N_2. Nizet's finding, therefore, appears fortuitous.

The velocity of the reaction is sufficiently high to make possible a rapid reduction of the methemoglobin formed when phenylhydrazine reacts with oxyhemoglobin. In the presence of atmospheric O_2, the reduced hemoglobin is reoxygenated and reacts with more phenylhydrazine. Thus the reaction of phenylhydrazine with oxyhemoglobin in the presence of oxygen and phenylhydrazine is cyclic as long as hemoglobin remains unmodified. However, as has been shown by Kiese and Seipelt (7) and Beaven and White (1), a green pigment forms during the course of the reaction. This results in permanent loss of O_2- and CO-combining power. In the absence of oxygen, the reaction of phenylhydrazine with methemoglobin does not result in any loss of CO-combining power and the green pigment is not formed. It would appear probable that several moles of phenylhydrazine could be decomposed by 1 mole of oxyhemoglobin in the presence of atmospheric oxygen but that the reaction is self-limited by the formation of the green pigment, and that the eventual total destruction of hemoglobin occurs in a manner similar to that reported by Lemberg, Legge, and Lockwood (8) in the reaction of oxyhemoglobin and ascorbic acid.

SUMMARY

1. Methemoglobin is reduced by phenylhydrazine with considerable velocity under anaerobic conditions.

2. In the reduction, 1 mole of methemoglobin is reduced and 2 moles of phenylhydrazine are oxidized to 2 moles of benzene and 2 moles of N_2.

3. The relatively high velocity of the reduction of methemoglobin by phenylhydrazine makes possible a cyclic reaction between oxyhemoglobin and phenylhydrazine.

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BIOSYNTHESIS OF THE PURINES

XI. STRUCTURE, ENZYMATIC SYNTHESIS, AND METABOLISM OF GLYCINAMIDE RIBOTIDE AND (α-*N*-FORMYL)-GLYCINAMIDE RIBOTIDE*

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The study of the *de novo* synthesis of the purine ring has been greatly facilitated by the complete knowledge of its elementary precursors (2–5) and by the finding that the reactions involved are carried out by soluble enzyme systems of pigeon liver (6, 7). Greenberg (8) has shown that inosinic acid (IMP) is a precursor of hypoxanthine in this system, indicating that ribotide compounds are involved as intermediates in the scheme. The recent isolation of two ribotide derivatives of glycinamide (1, 9), which are intermediates in the biosynthesis of inosinic acid, suggests that the step whereby the ribose is attached occurs early in the sequence of reactions.

This paper is concerned with the enzymatic preparation of these two aliphatic ribotides, glycinamide ribotide (GAR) and (α-*N*-formyl)-glycinamide ribotide (FGAR), and with the proof of structure of these two compounds by chemical analysis. A report is also made of the substrate requirements for the synthesis of these compounds and for their conversion to inosinic acid. It has been found that 5-phosphoribosyl pyrophosphate (PRPP) participates as the donor of the ribotidyl moiety of these intermediates and that glutamine and adenosine triphosphate (ATP) are involved in the synthesis of glycinamide ribotide from glycine.

The antibiotic, L-azaserine, which exerts an inhibitory effect on the growth of Crocker mouse sarcoma 180 (10) and significantly reduces the incorporation of C^{14}-formate in tumor tissues (11), has been shown to have

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a specific action on the enzymatic system responsible for the synthesis of inosinic acid from its precursors.

EXPERIMENTAL

Methods—Glycine was determined by the method of Alexander *et al.* (12) after hydrolysis of the aliphatic ribotide in 2.5 N H_2SO_4 for 90 minutes at 150°. Formic acid was determined by the method of Grant (13) after hydrolysis of the ribotide in 1 N NaOH for 50 minutes at 100°. Hydrolysis of the ribotides in 2 N H_2SO_4 for 90 minutes at 150° quantitatively liberated amide nitrogen as NH_3, which was subsequently determined colorimetrically with the Nessler reagent. Total nitrogen was determined by the Kjeldahl procedure. Inorganic phosphorus was determined by the method of Fiske and Subbarow (14). Organic phosphorus was determined by this same method after digestion of the sample in 10 N H_2SO_4 for 90 minutes at 150°.

The orcinol method for the determination of pentose (15) was modified to produce equivalent color values for both the standard and unknown compounds tested. Although adenosine-5′-phosphate is a suitable standard for the analysis of most purine ribonucleotides, it was not satisfactory as such for the determination of the pentose of the aliphatic ribotides. The rate of color development and the final intensity of the color of adenosine-5′-phosphate in the orcinol reaction were greater than that shown by samples of these ribotides known by other analyses to be of equal concentration. Hydrolysis of all samples to ribose-5-phosphate in 1.3 N HCl for 40 minutes at 100° immediately prior to color development eliminated these difficulties.

Radioactive Assay for Aliphatic Ribotides—The enzymatic reaction was stopped by heating the vessels at 100° for ½ minute. The incubation mixture was chilled in ice and acidified with 0.05 ml. of 4 N HCl. This material was placed on a Dowex 50 ammonium column (0.8 × 6.0 cm.), and the initial eluate was collected in a 10 ml. graduate cylinder. The tube was rinsed with 2 ml. of 0.05 M ammonium formate buffer, pH 3.3. These washings were passed through the resin and collected together with the initial eluate. The resin was then washed further with separate small portions of the ammonium formate buffer until a total volume of 6.0 ml. was collected. By this procedure, both GAR and FGAR passed through the column, but labeled glycine was retained. A suitable aliquot was then plated in duplicate, evaporated to dryness under an infra-red lamp, and assayed for radioactivity. When a resolution of the two aliphatic ribotides was desired, the initial eluate and buffer washes were made strongly alkaline with a few drops of 10 N KOH and placed on a Dowex 1 acetate column (0.8 × 6.0 cm.). Elution was carried out with 0.05 M ammonium acetate as described in more detail under "Results." The elution of radioactive

GAR and FGAR in the order named was followed by plating of a small aliquot from each tube of the eluate.

Assay for Synthesis of Inosinic Acid de Novo—The method of Schulman, Sonne, and Buchanan (7) was employed for the determination of the synthesis of inosinic acid from labeled elementary precursors or from aliphatic ribotide intermediates.

Materials—PRPP was obtained as the magnesium salt by a slight modification of the procedure of Remy, Remy, and Buchanan (16). Barium ribose-5-phosphate was obtained by the procedure of Khym, Doherty, and Cohn (17). 3-Phosphoglyceric acid was purchased from the Schwarz Chemical Laboratories, Inc. The above compounds were all converted to their sodium salts either by passage through Dowex 50 sodium or by direct metathesis with Na_2SO_4. L-Azaserine was kindly supplied by Dr. A. Moore of Parke, Davis and Company and The Mellon Institute. Crystalline ATP (disodium salt) was obtained from the Pabst Brewing Company. L-Glutamine and L-aspartic acid were products of the Mann Research Laboratories. Glycine-1-C^{14}, $HC^{14}OONa$, and $NaHC^{14}O_3$ were purchased from Tracerlab, Inc.

RESULTS AND DISCUSSION

In early attempts to accumulate intermediates concerned with the biosynthesis of inosinic acid *de novo*, the 0 to 45 per cent ethanol fraction of pigeon liver extract was incubated together with substrate quantities of the purine elementary precursors, glycine, glutamine, formate, and bicarbonate, as well as ribose-5-phosphate, ATP, and 3-phosphoglyceric acid (3-PGA). Aspartic acid, the donor of nitrogen atom 1 of the purine ring (5), was omitted to favor the accumulation of possible intermediates. After incubation with glycine-1-C^{14} as the tracer molecule, the reaction mixtures were deproteinized and chromatographed on a Dowex 1 chloride column. The results of these preliminary experiments indicated the presence of radioactive components which were eluted readily with 0.003 N HCl and which possessed no selective absorption in the wave-lengths between 230 and 290 mμ. At this time it was noted that the antibiotic, L-azaserine, exerted a pronounced inhibitory effect on the synthesis of IMP *de novo* (18) and that, concurrent with this inhibition, there was an enhancement of the accumulation of the unknown compounds previously mentioned. The use of this selective metabolic inhibitor, together with the development of an improved method of isolation of the unknown compounds (see below), made it possible to obtain these labeled products in sufficient quantity and purity to determine their structure and to demonstrate their rôle as intermediates in inosinic acid biosynthesis.

In the procedure as it was finally developed, the products of reaction

were first chromatographed on a Dowex 50 ammonium column and then on a Dowex 1 acetate column. In Fig. 1 is presented a chromatogram from a column of Dowex 1 acetate, showing the elution of the two major radioactive compounds formed in the presence and absence of azaserine. The first component, which was subsequently identified as GAR and which could be labeled only from radioactive glycine, was always found in much smaller proportions than the second component. This latter compound,

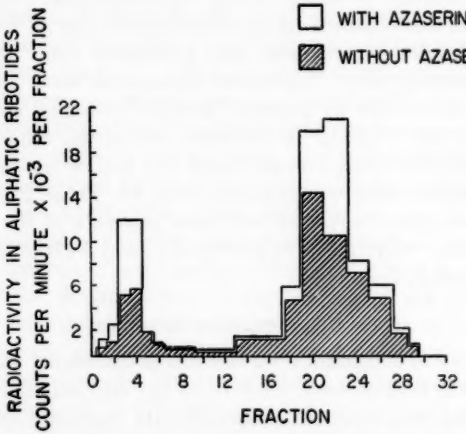


FIG. 1. Chromatographic separation of aliphatic ribotides; effect of L-azaserine on the accumulation of these compounds *in vitro*. The contents of the first vessel were glycine-1-C^{14} (40 μmoles), glutamine (40 μmoles), ribose-5-phosphate (40 μmoles), $KHCO_3$ (40 μmoles), Na formate (40 μmoles), ATP (4 μmoles), 3-phosphoglycerate (40 μmoles), 150 mg. of 0 to 45 ethanol fraction of pigeon liver extract, 1 ml. of 0.04 M phosphate buffer, pH 7.4, containing 10 μmoles of $MgCl_2$. Final volume, 4 ml. The contents of the second vessel were the same as the first vessel but with 8 μmoles of L(+)-azaserine added. The analysis of the compounds was the same as indicated in the text except that resin columns were 12 cm. long instead of 6 cm.

which was later identified as FGAR, could be labeled from both radioactive glycine and radioactive formate. In some experiments a third component could be eluted in small amounts after the GAR and just prior to the appearance of the FGAR. This material could also be labeled with formate and glycine, and in all probability was another form of FGAR which was produced as an artifact in the isolation procedure.

It may be seen that the accumulation of both compounds is enhanced by the presence of L-azaserine. In some experiments the increase in aliphatic ribotides as a result of inclusion of azaserine was 4- to 5-fold, while in others the increment was more conservative. This was probably related

to the variation of the ability of the uninhibited extracts to synthesize inosinic acid from glycine.

Experiments were carried out to determine whether these compounds were intermediates in the biosynthesis of inosinic acid. It is seen in Table I that both GAR and FGAR are excellent precursors of IMP in the complete system, and that the incorporation of their radioactivity into IMP was unaffected by the presence of a bank of unlabeled glycine. This latter information demonstrated that the compounds were not first degraded to glycine before being utilized for IMP formation. In addition,

TABLE I

Comparison of Utilization of Radioactive Glycine and Glycine Derivatives for Inosinic Acid Synthesis

Substrates	Specific activity of inosinic acid	Relative utilization of substrates for inosinic acid synthesis
	c.p.m. per μmole	
Glycine-1-C^{14}	20.8	1
Glycinamide ribotide-1-C^{14}	26.9	
" + glycine	27.6	1.3
Formylglycinamide ribotide-1-C^{14}	39.2	
" + glycine	45.1	2.0
Component 3*-C^{14}	0.4	0.01

*See the text.

All vessels contained L-glutamine (4.5 μmoles); L-aspartic acid (5 μmoles); sodium formate (12 μmoles); potassium bicarbonate (24 μmoles); 3-phosphoglyceric acid (6 μmoles); 0.3 ml. of boiled juice and 0.2 ml. of 0.04 M phosphate buffer, pH 7.4, containing 2 μmoles of $MgCl_2$; 0.3 ml. of enzyme (0 to 45 per cent ethanol precipitate) equivalent to 0.6 ml. of original pigeon liver extract. The total volume equals 1.0 ml. All radioactive substrates were added at a level of 0.4 μmole. When non-radioactive glycine was added, it was present in concentrations of 15 μmoles per ml. Incubation time, 60 minutes; temperature, 38°.

chemical degradation of the purine base (19) formed biosynthetically from a sample of formyl-labeled FGAR revealed that essentially all the C^{14} was located in carbon atom 8.

It is of interest that the third compound, which was isolated from the chromatogram associated with FGAR as mentioned above, had no activity as a substrate for inosinic acid biosynthesis. This is in contrast to results obtained by Goldthwait *et al.* (20), who have isolated a second form of FGAR and reported it to have activity in their enzymatic system. The non-utilization of this third component as a substrate by our enzymatic system might lead one to suspect that it is a product of mutarotation and perhaps, therefore, the α form of the ribotide.

In the further metabolic reactions of the aliphatic ribotides both glutamine and aspartic acid were required to convert FGAR to IMP (Table II). As seen in the final row of Table II, L-azaserine inhibits the conversion of FGAR to IMP. This would be expected in view of the fact that FGAR accumulated in the presence of this antibiotic during reactions of glycine directed toward purine biosynthesis.

Enzymatic Synthesis and Isolation of FGAR

Preparation of Enzyme System—Pigeon liver extract was prepared as previously described (7). An equal volume of 90 per cent ethanol was added to the extract dropwise, the solution stirred at −18° for 10 minutes,

TABLE II

Nitrogenous Substrates Required for Conversion of Formylglycinamide Ribotide to Inosinic Acid

Vessel	Nitrogenous substrate		Azaserine	Inosinic acid formed
	Glutamine	Aspartic acid		
	μmoles	*μmoles*	*μmoles*	*μmole*
1				0.003
2	15			0.030
3		15		0.027
4	15	15		0.160
5	15	15	2.8	0.048

All vessels contained in a final volume of 2.4 ml. sodium formate (30 μmoles); potassium bicarbonate (15 μmoles); 3-phosphoglyceric acid (15 μmoles); 0.3 ml. of 0.04 M phosphate buffer, pH 7.4, containing 3 μmoles of $MgSO_4$; the barium salt of formylglycinamide ribotide-1-C^{14} (0.4 μmole); 0.5 ml. of enzyme ("0 to 45" ethanol fraction) equivalent to 1 ml. of original pigeon liver extract.

and the protein collected by centrifugation. The precipitate was stored in this form at −18° and used usually within a week.

Incubation Procedure—Each vessel contained, in a final volume of 50 ml., the following quantities of materials expressed in micromoles: glycine-1-C^{14} (225), L-glutamine (240), L-azaserine (72), sodium formate (170), sodium 3-phosphoglycerate (720), sodium ribose-5-phosphate (320), potassium bicarbonate (900), disodium ATP (45), sodium phosphate buffer (500), pH 7.4, KCl (2000), $MgCl_2$ (160), and the precipitated enzyme equivalent to 50 ml. of pigeon liver extract. This reaction mixture was incubated for 90 minutes at 37° and then deproteinized with 3 ml. of 30 per cent trichloroacetic acid (TCA). The precipitate was removed by centrifugation.

Isolation of FGAR by Chromatography and Purification of Barium Salt—The supernatant solution from the deproteinized incubation mixture was placed on a Dowex 50 ammonium column (2.5 × 7.5 cm.). The protein precipitate was washed with 25 ml. of 0.05 M ammonium formate buffer, pH 3.35, and the washings were added to the column. Finally, another 15 ml. of the buffer were used to wash the column. The above three eluates, which were combined, contained all the FGAR and practically no glycine.

The solution was then adjusted with 10 N KOH to pH 10 and placed on a Dowex 1 acetate column (2.5 × 7.5 cm.). The column was then eluted with 0.05 M ammonium acetate buffer, pH 5.3. Fractions of 10 ml. were collected every 15 minutes, and the elution of radioactive compounds was followed by the method described above. A small amount of radioactivity representing the GAR was eluted between 100 and 200 ml., while the bulk of the radioactivity, the FGAR, was eluted between 300 and 500 ml. The eluate containing the FGAR was lyophilized to 10 ml.

4 volumes of ethanol were added, and the solution was chilled to −15°. By a process of fractional precipitation of barium salts, the more insoluble phosphate impurities, *i.e.* inorganic and ribose-5-phosphate, were precipitated and separated by centrifugation from the radioactive material (FGAR) which remained in solution. Under the conditions of this preparation, 300 μmoles of barium acetate were initially added and followed by smaller portions until the radioactivity just began to precipitate. These impurities were removed by centrifugation. 300 μmoles of barium acetate were then added at −15° to the clear supernatant solution, and the barium salt of FGAR which precipitated was collected by centrifugation. Approximately 90 per cent of the radioactivity was recovered in this crude barium salt of FGAR. This could be further purified by extraction of the precipitate with small amounts of water until the FGAR was all in solution and by reprecipitation of the ribotide by addition of 4 volumes of ethanol. This material was used for enzymatic studies. The yield was about 60 μmoles.

However, for material which was used for chemical analysis, a further purification procedure was applied to this barium salt. It was dissolved in water, made slightly alkaline, and placed on a Dowex 1 acetate column (1 × 10 cm.). Two radioactive components were eluted with 0.05 M ammonium acetate buffer, pH 5.35. The second component, which comprised 90 per cent of the total radioactivity, was collected, lyophilized to 1 ml., and treated with 35 μmoles of barium acetate and 4 volumes of ethanol. The precipitate was washed with ethanol and ether and dried *in vacuo*. This material was used for the FGAR analysis.

Enzymatic Synthesis and Isolation of GAR

Preparation of Enzyme System—The 0 to 15 per cent ethanol fraction of pigeon liver was prepared as previously described. To prepare the 15 to 30 per cent ethanol fraction, pigeon liver was homogenized in a blendor and then centrifuged in the Servall SS-1 centrifuge at 10,000 r.p.m. for 10 minutes. To the supernatant solution was added 0.2 volume of 90 per cent cold ethanol, and the precipitate was removed by centrifugation at −7°. To the supernatant solution was added 0.25 volume of 90 per cent cold ethanol. The precipitate, which was collected by centrifugation at −18°, was dissolved in a minimal amount of water and lyophilized. This "15 to 30" ethanol fraction remains active when stored at −5° for at least 3 months. To prepare this enzyme for use, 25 mg. of the lyophilized "15 to 30" ethanol fraction were dissolved per ml. of 0.05 M tris(hydroxymethyl)aminomethane (Tris), pH 8.8, and treated for 1 hour with 25 mg. of Dowex 1 chloride resin by stirring at 2°. This treatment removes formylation cofactors from the enzyme (9).

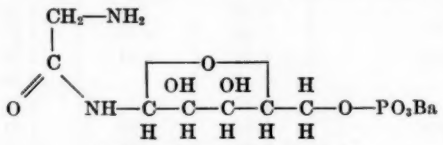
Incubation Procedure—Each vessel contained the following quantities of materials expressed as micromoles: glycine-1-C^{14} (1000); L-glutamine (1000); $KHCO_3$ (3000); disodium ATP (600); sodium ribose-5-phosphate (600); $MgCl_2$ (600); K_2HPO_4 (1000); 16 ml. of 0.25 M Tris, pH 8.8; 1500 mg. of Dowex-treated "15 to 30" ethanol fraction of pigeon liver dissolved in 60 ml. of 0.05 M Tris, pH 8.8; 260 mg. of "0 to 15" ethanol fraction of pigeon liver dissolved in 20 ml. of 0.05 M Tris, pH 8.8. The total volume of the reaction mixture was 140 ml. The vessels were incubated 30 minutes at 37°, chilled, and then deproteinized with 20 ml. of 30 per cent TCA. The insoluble material was removed by centrifugation, and the supernatant solution used for the isolation of GAR by resin chromatography.

Isolation of GAR by Chromatography and Purification of Barium Salt—The procedure used for the isolation of GAR was essentially identical to that employed for FGAR, except in the following details. The dimensions of the Dowex 50 ammonium column were 2.5 × 17.5 cm., and those of the Dowex 1 acetate column 2.5 × 15 cm. GAR was eluted from the latter after 200 ml. of 0.025 M ammonium acetate buffer, pH 5.35, had passed through the column. The barium salt of GAR obtained upon precipitation by 4 volumes of ethanol from the lyophilized eluate was sufficiently pure for use in enzymatic studies.

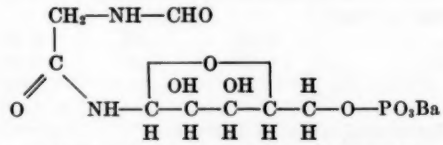
However, for analytical studies, further purification was necessary. 20 μmoles of the crude barium salt were dissolved in 5 ml. of H_2O, made alkaline with 2 ml. of 5 N NH_4OH, and placed on a Dowex 1 bromide column (1 × 10 cm.). The column was washed with water until the effluent was neutral and then eluted with 0.001 N HBr. The GAR was located in an early fraction which was lyophilized to 1 ml. 40 μmoles of barium bromide

and 4 volumes of ethanol were added, and the solution was chilled at −15° overnight. The precipitate which formed was purified by reprecipitation as described above and used as such for analyses.

Chemical Analyses of GAR and FGAR—On the basis of chemical analyses to be reported in this section, the following formulas have been considered as representing the correct structures of the two compounds isolated as barium salts in the enzymatic reactions of pigeon liver. Goldthwait, Peabody, and Greenberg (9) have proposed essentially these structures from metabolic considerations and from preliminary analytical data. They were, however, not able to prepare compounds of sufficient purity to obtain chemical analysis of hydrolytic products corresponding with the expected theoretical values. Moreover, they have never reported the isolation of their compounds in solid form to permit estimation of the absolute purity of their products or to exclude the presence of undetected groups.



Glycinamide ribotide (GAR)



(α-*N*-Formyl)-glycinamide ribotide (FGAR)

In Table III are reported data obtained (*a*) by the electrometric titration of the compounds and (*b*) by the chemical analysis of specific groups produced upon hydrolysis. These data are expressed in terms of the micromoles of substances determined or ionizable groups titrated per given quantity of barium salt of the ribotide. When values are converted to molecular ratios (glycine taken as unity), it may be seen that the various components of each compound are present in the relative amounts expected by theory.

In Table III the quantities of samples used for analysis should have contained 2.37 and 2.22 μmoles of GAR and FGAR per mg. of barium salt, respectively, if the samples weighed had been pure. When comparison of these values is made to the other analyses on the compounds, it may be seen that GAR and FGAR are about 71 and 85 per cent pure, respectively.

Other Properties of GAR and FGAR—In addition to the chemical analyses presented above, several other properties of the compounds support

the structural formulas assigned to them. The phosphate group of the aliphatic ribotides has the same relative stability toward acid hydrolysis as does adenosine-5′-phosphate. This, in addition to the fact that the ribose-phosphate moiety is derived metabolically from PRPP (see below), is strong evidence that these intermediates are 5-phosphoribosyl compounds. That these compounds may be enzymatically converted into inosine-5′-phosphate (IMP) is further indication of their structure.

From a metabolic point of view this phosphoribosyl moiety must be

TABLE III

Analysis of Glycinamide Ribotide (GAR) and Formylglycinamide Ribotide (FGAR)

Method of analysis*	Ba salt of GAR	Molecular ratio	Ba salt of FGAR	Molecular ratio
	μmoles per mg.	*(glycine = 1)*	*μmoles per mg.*	*(glycine = 1)*
Glycine	1.67	1.00	1.89	1.00
Formate	0.07		2.11	1.11
Pentose	1.64	0.98	1.81	0.96
Organic phosphate	1.74	1.04	2.03	1.07
Inorganic "	0.13		0.06	
Total phosphate	1.87		2.09	
Acid labile nitrogen	1.64	0.98	1.91	1.01
Total nitrogen	3.23	1.93	3.78	1.90
C^{14}	1.50	0.90	1.83	0.97
Secondary phosphate hydrogen ion	1.82	1.06	2.20	1.16
Amino hydrogen ion	1.82	1.06	0.00	0.00
Dry weight†	2.37		2.22	

* See the methods of analysis in "Experimental."

† 1 mg. of a pure compound should contain the indicated number of micromoles of ribotide. Molecular weight of the barium salt of FGAR equals 450 and of the barium salt of GAR equals 422. For analysis of the compounds 2.07 mg. of the barium salt of FGAR were dissolved in 4 ml. of water, and 8.18 mg. of the barium salt of GAR were dissolved in 5 ml. of water.

linked to the molecule through a nitrogen atom which eventually becomes N_9 of the purine ring. Undoubtedly this nitrogen is the one of the two nitrogens of either compound which is readily hydrolyzed to ammonia in acid or alkaline solution. The lability of this nitrogen atom to acid or alkaline hydrolysis suggests that it is present in the molecule in amide linkage.

The fact that FGAR does not react with the Pauly diazo reagent for imidazoles is suggestive but not conclusive evidence that it exists in a non-cyclized form. It is of further note that these compounds absorb only weakly in wave-lengths of light below 230 mμ and have no significant absorption in the higher ultraviolet or visible regions.

We have reexamined the problem of the position of the formyl group in FGAR. Evidence from experiments in this laboratory demonstrated that it is present on the amino nitrogen and not the amide nitrogen, as previously reported by Goldthwait *et al.* (20). This evidence consists of the following: (*a*) In contrast to the report of Goldthwait *et al.* (20), GAR gives a positive ninhydrin color reaction with a color value approximately equal to that of glycine when the reaction is carried out according to the

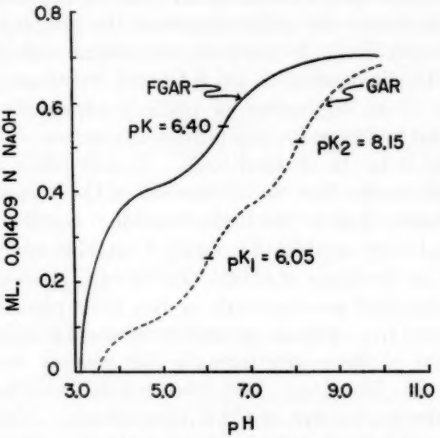


FIG. 2. Titration curves of glycinamide ribotide and (α-*N*-formyl)-glycinamide ribotide. (*a*) 2.31 mg. of the barium salt of purified GAR were dissolved in water and placed on a Dowex 50 H^+ column (0.8 × 2 cm.). As determined by radioactive measurement, the equivalent of 2.08 mg. of the above material was collected for titration with the glass electrode with 0.01409 N NaOH. (*b*) 1.71 mg. of the barium salt of purified FGAR were dissolved in water, placed on a Dowex 50 H^+ column (0.8 × 2 cm.), and quantitatively recovered in the effluent. The material in the effluent was titrated with the glass electrode with 0.01409 N NaOH. Both curves were corrected for the titration of water.

method of Cocking and Yemm (21). However, FGAR does not give any reaction with this reagent. This would be expected if the formyl group is present on the amino nitrogen. (*b*) Studies with paper electrophoresis (0.05 M ammonium acetate, pH 7.0, 90 minutes, 16 volts per cm.) demonstrate that FGAR has a mobility twice that of GAR. This again suggests that the amino group of FGAR is substituted and hence uncharged. (*c*) The most convincing evidence for this point of view comes, however, from titration curves of the purified compounds. As shown in Fig. 2 and in Table III, approximately 1 equivalent of hydrogen ion is titrated as the secondary hydrogen of phosphate for both compounds. However, only

GAR has a group titrating in the vicinity of the amino range, *i.e.* pH 8.0. According to the determinations shown in Fig. 2, the pK of the secondary hydrogen ion of phosphate is 6.05 for GAR and 6.40 for FGAR. The pK of the dissociation of hydrogen ion of the amino group of GAR is 8.2.

The results of the titrations of GAR and FGAR indicate that GAR precipitates as the barium salt of the free base rather than as the hydrobromide. Upon passage through Dowex 50 hydrogen form the barium salt of FGAR, which has its amino group formylated, yields essentially 2 equivalents of hydrogen ion which represent the primary and secondary phosphate hydrogen ions. In contrast, the barium salt of GAR yields 2 equivalents of H^+, dissociating at pK 6.1 and 8.2 without any evidence of the dissociation of an equivalent of primary phosphate hydrogen. In all probability this hydrogen ion was utilized in reaction with the free amino group to convert it to the charged form. This evidence of internal neutralization would require that the barium salt of GAR would have precipitated as such rather than as the hydrobromide. Insufficient amounts of the pure material were available to obtain a bromide analysis.

Requirements for Synthesis of GAR—Goldthwait, Peabody, and Greenberg (9) have described an enzymatic system from pigeon liver in which GAR is synthesized from glycine, glutamine, ribose-5-phosphate, and ATP. The participation of these substrates in this general reaction has been corroborated in this laboratory, and these results extended to determine in more detail the mechanism of GAR biosynthesis. The first object of these studies was to determine the exact nature of the compound responsible for the addition of the phosphoribosyl moiety of glycinamide ribotide. Recent work has shown that ATP and ribose-5-phosphate react to form PRPP, which may then condense directly with a number of purine (16, 22) and pyrimidine (22) bases to form the corresponding ribotides. It was, therefore, desirable to test this compound (PRPP) as the effective agent in the step at which ribose is attached during purine biosynthesis *de novo* (*i.e.*, the conversion of glycine to glycinamide ribotide).

Since unfractionated preparations of pigeon liver are capable of converting ribose-5-phosphate and ATP to PRPP, it was impossible to determine the unique rôle of this latter compound in the reaction until the enzyme responsible for PRPP synthesis was removed. From previous studies it had been shown that this enzyme occurred in a fraction of pigeon liver which precipitated below 15 per cent ethanol. Experiments were therefore carried out in which a fraction precipitating between 15 and 45 per cent ethanol was utilized as the enzyme preparation for the study of the conversion of glycine to glycinamide ribotide. The "15 to 45" ethanol fraction was relatively free of the PRPP-synthesizing system, but could be still further depleted by dialysis of the enzyme system against water and by

removal of the precipitate formed. In order to remove nucleotide and transformylating cofactors, the enzyme preparation was treated with Norit (9). When ribose-5-phosphate and ATP as well as glutamine and glycine were used as substrates of the reaction in the presence of this enzyme preparation, little or no synthesis of GAR was observed (Table IV). Moreover, PRPP could not substitute for ribose-5-phosphate and ATP. Upon recombination of various substrates, however, it was found that good synthesis could be obtained if both ATP and PRPP were included with glutamine and glycine. It is, therefore, evident that in the original system ATP had a dual rôle, one of pyrophosphorylating ribose-5-phosphate and a second function which at present is not fully understood. These experiments show, however, that PRPP is the active substrate responsible for the donation of the phosphoribosyl moiety. Since publication of our preliminary communication on the rôle of PRPP and ATP in this system, Goldthwait *et al.* (23) have confirmed these observations and, further, have implicated a rôle for 5-phosphoribosylamine in these reactions.

TABLE IV

Involvement of PRPP and ATP in Synthesis of GAR

Addition to basic system	GAR synthesized
	μmoles
None	0.00
Ribose-5-phosphate, 2 μmoles	0.02
ATP, 2 μmoles	0.04
Ribose-5-phosphate + ATP, 2 μmoles each	0.07
PRPP, 2 μmoles	0.13
PRPP + ATP, 2 μmoles each	0.43

The basic system included 5 μmoles of glycine-1-C^{14}, 5 μmoles of L-glutamine, 7 μmoles of $NaHCO_3$, 2 μmoles of L-azaserine, 0.3 ml. of 0.03 M phosphate buffer, pH 7.4, containing 0.13 M KCl and 0.01 M $MgCl_2$, and 10 mg. of dialyzed, Norit-treated 15 to 45 per cent ethanol fraction of pigeon liver extract; incubated 45 minutes at 38°.

Glutamine has been shown to be the only compound capable of donating the amide nitrogen of GAR. Substitution by other compounds such as asparagine, ammonium ions, and aspartic and glutamic acids has been tried without success. In a similar manner, glycinamide itself would replace the requirement for neither glutamine nor glycine and in fact is not an intermediate in the reaction (18).

SUMMARY

Glycinamide ribotide and (α-*N*-formyl)-glycinamide ribotide, which are formed by an enzyme system of pigeon liver synthesizing inosinic acid,

have been isolated, purified, and characterized by chemical analysis. Phosphoribosyl pyrophosphate, ATP, and glutamine are involved in the synthesis of glycinamide ribotide from glycine. Azaserine is a potent inhibitor of inosinic acid biosynthesis *in vitro* and results in an increased accumulation of these ribotides.

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THE STRUCTURE OF GLYCINAMIDE RIBOTIDE*

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The occurrence of glycinamide ribotide and its formyl derivative and the involvement of these compounds in the synthesis of inosinic acid have been discussed previously (1–4). This paper describes a method of isolating and purifying glycinamide ribotide and presents evidence that it has the structure *N*-glycyl-5-phosphoribofuranosylamine.

EXPERIMENTAL

Materials—The pigeon liver extract preparation and the substrates required for the synthesis of glycinamide ribotide have been described previously (1–4). Intestinal phosphatase, purified grade, was obtained from Pentex, Inc., Kankakee, Illinois. Adenosine-5′-phosphate was obtained from the Ernst Bischoff Company, Inc., Ivoryton, Connecticut. Adenosine-3′-phosphate was obtained from the Schwarz Laboratories, Inc., New York.

Methods—For paper chromatography Whatman No. 1 paper was washed with 2 N acetic acid and then with 10^{-4} M ethylenediamine sodium tetraacetate. The following solvent systems were employed for paper chromatography (all ratios are in volume proportions): (1) *n*-butanol-17.6 N acetic acid-water (2:1:1), (2) isopropanol-water (70:30) in an atmosphere of ammonia (0.35 ml. of concentrated ammonium hydroxide per liter of jar volume), (3) propanol-water (70:30), (4) 1.0 M ammonium acetate, pH 7.5-95 per cent ethanol (30:75), (5) 95 per cent ethanol-0.025 M potassium borate, pH 8.2 (60:40), (6) 100 gm. of phenol plus 39.5 gm. of water, (7) tertiary amyl alcohol saturated with 0.05 M potassium phthalate, pH 6.0, and (8) 1.0 M ammonium acetate, pH 7.5-95 per cent ethanol (30:60). In

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the case of Solvent 7, the chromatographic paper was dipped in the potassium phthalate buffer and dried prior to the application of compounds.

Radioactive compounds were located on the paper chromatograms with an end window monitor. Phosphate compounds were detected with the reagent of Isherwood and Hanes (5, 6) by using the ultraviolet irradiation procedure of Bandurski and Axelrod (7) to develop the color. Amino acids were located by the ninhydrin reagent (0.1 gm. of ninhydrin per 100 ml. of *n*-butanol saturated with 1 per cent acetic acid), reducing sugar by reaction with aniline phthalate (8), and non-reducing sugar by the periodate reaction (9).

For anion exchange procedures Dowex 1 (4 per cent cross-linked, 250 to 400 mesh) in the formate, acetate, or chloride form was used. For cation exchange procedures Dowex 50 (10 per cent cross-linked, 50 to 100 mesh) was used. The radioactivity of fractions eluted from resin columns was measured in glass planchets as previously described (2, 3).

The method of analysis for glycinamide ribotide has been described (2, 3). Total nitrogen was estimated by digestion with sulfuric acid and by direct nesslerization (10). Ammonia liberated by acid hydrolysis of glycinamide ribotide was determined, after diffusion (11), by Nessler's reagent. Inorganic phosphate was determined by the Gomori modification (12) of the method of Fiske and Subbarow (13). Total phosphate was determined after wet ashing with 0.2 ml. of 70 per cent perchloric acid for 30 minutes. Pentose was determined by the orcinol method (14) by heating for 45 minutes at 100°, or by measuring the consumption of periodate spectrophotometrically (15). The adenosine-5′-phosphate used as a standard for the orcinol method consumed 1.0 mole of periodate per mole of organic phosphate and had a molecular extinction coefficient at 260 mμ of 15,800 (16). Reducing sugar was determined by the Park and Johnson (17) modification of the ferricyanide method of Folin and Malmros (18). Ribose-5-phosphate was used as a standard. Samples which had been hydrolyzed in acid were brought to neutrality with NaOH before analysis. Glycine was determined by a colorimetric ninhydrin method (19) (Method 1) and by a modification of the more specific method of Alexander *et al.* (20) (Method 2) in which formaldehyde is determined directly in the ninhydrin reaction mixture.[1] A Beckman DU spectrophotometer was used for all colorimetric measurements.

Preparation of Glycinamide Ribotide—Glycinamide ribotide labeled with C^{14} was synthesized enzymatically by adding the following reagents in a final volume of 102 ml.: 3.55 gm. of Dowex 1-treated and dialyzed extract of pigeon liver acetone powder (2, 3) as a lyophilized powder, 0.92 mmole of

[1] The authors are grateful to Dr. Lothar Jaenicke, Marburg, Germany, for providing us with the details of this procedure before publication.

glycine-1-C^{14} containing 3×10^6 c.p.m. per mmole, 0.32 mmole of ATP,[2] 0.44 mmole of ribose-5-phosphate, 2.48 mmoles of 3-phosphoglycerate, 0.89 mmole of glutamine, 1.13 mmoles of $MgCl_2$, and 3.55 mmoles of Tris buffer at pH 8.0. Equal parts of the mixture were transferred to four 125 ml. Warburg flasks and incubated 33 minutes at 38° under nitrogen. The reaction mixture was cooled to 0°, and 20 per cent TCA was added to a final concentration of 5 per cent. The resulting precipitate was centrifuged and washed three times with a total of 140 ml. of 5 per cent TCA. The supernatant solution and the washings were combined, and the TCA was removed by extraction with ether. The remaining aqueous solution (260 ml.) was neutralized and assayed for glycinamide ribotide (2, 3). The total C^{14} activity incorporated into the glycinamide ribotide fraction was found to be 381,000 c.p.m. or the equivalent of 127 μmoles of C^{14}-glycine. Glycinamide ribotide was separated from inorganic phosphate, ATP, and PGA by fractionation with barium (2, 3). The solution of glycinamide ribotide was adjusted to pH 8.2, and 15.8 ml. of 0.5 M barium acetate solution was added. The precipitate was dissolved in 0.1 N hydrochloric acid and reprecipitated by addition of dilute ammonium hydroxide. This procedure was repeated once. The combined supernatant solutions were adjusted to pH 3 with concentrated formic acid and passed through a column of Dowex 50, NH_4^+ resin (1.54 sq. cm. × 18.5 cm.). The column was washed with 0.03 M ammonium formate at pH 3.4 until orcinol-reacting material no longer was eluted (45 ml.). The washings were combined with the main effluent (total volume 440 ml.), and, after neutralization, the solution was passed through a column (2.7 sq. cm. × 3.8 cm.) of acid-washed Norit A charcoal. The charcoal column then was washed with 25 ml. of water, and the wash combined with the main effluent. This solution was adjusted to pH 9 with ammonium hydroxide, diluted to 2 liters, and passed through a Dowex 1 column (0.9 sq. cm. × 26 cm., formate form). All of the orcinol-reacting material was retained on the column. The compounds were eluted with ammonium formate buffers (Fig. 1). The main fraction of radioactivity, eluted at pH 6.5, contained 258,000 c.p.m. (85.9 μmoles of glycinamide ribotide), while a minor fraction which was eluted at pH 4.0 with 0.1 M ammonium formate solution contained 25,300 counts and corresponded to formylglycinamide ribotide (1–3). The major component exhibited a constant ratio of radioactivity to orcinol-reacting material in each fraction. This component was lyophilized to dryness to reduce the quantity of ammonium formate, diluted to 150 ml., adjusted to pH 9 with am-

[2] The following abbreviations are used: AMP-3, adenosine-3′-phosphate; AMP-5, adenosine-5′-phosphate; ATP, adenosine-5′-triphosphate; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; DNP, 2,4-dinitrophenyl; FDNB, 1-fluoro-2,4-dinitrobenzene; PGA, D-3-phosphoglycerate.

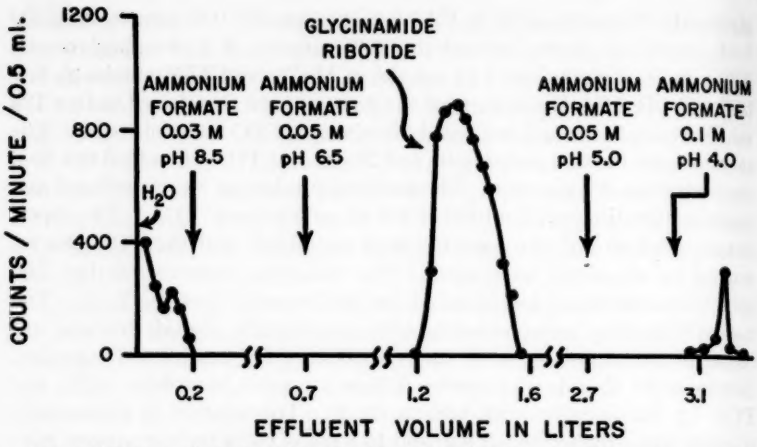


FIG. 1. Elution of glycinamide ribotides from Dowex 1 formate column. The preparation and prior treatment of glycinamide ribotide are described in the text. No additional radioactive compounds were eluted with 1.0 M ammonium formate, pH 4.0.

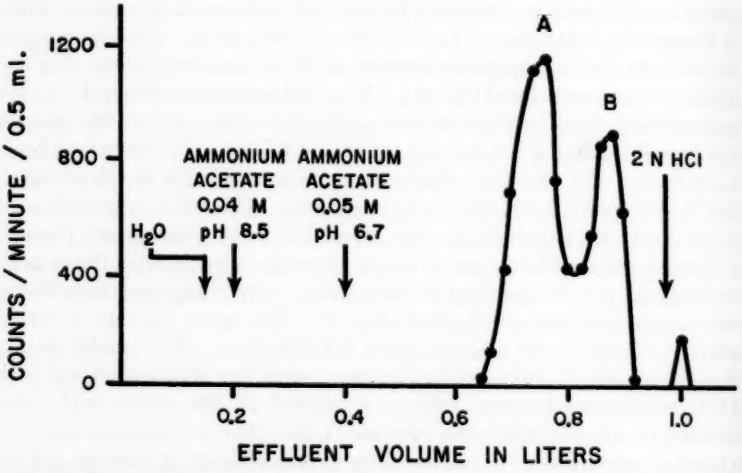


FIG. 2. Elution of glycinamide ribotides from Dowex 1 acetate column. The glycinamide ribotide used was isolated from the Dowex 1 formate column (see the text and Fig. 1).

monium hydroxide, and passed through a second Dowex 1 column (acetate form, 0.64 sq. cm. × 35 cm.). The column was eluted as indicated in Fig. 2.

At pH 6.7 a partial separation of the major component into two bands of radioactivity (A and B) was obtained. Again the ratio of radioactivity to pentose concentration in each fraction of these bands was constant. Portions of the two bands were used for the paper chromatography. The component first eluted from the column was designated glycinamide ribotide A (Table I). The material represented by band A was adjusted to pH 9, diluted with an equal volume of water, and adsorbed onto a small Dowex 1 column (chloride form, 0.64 sq. cm. × 2.0 cm.). The column was washed with 400 ml. of water and then with 15 ml. of 0.03 M NaCl containing 0.01 N HCl. 43 μmoles of glycinamide ribotide, free of ammonia and acetate, were eluted with the NaCl-HCl solution in a volume of 32 ml. This fraction was used for the analyses reported in Table II, for the acid hydrolysis studies, for the formation of glycinamide riboside and DNP-glycinamide ribotide, and for the preparation of the calcium salt of the ribotide. The calcium

TABLE I

Paper Chromatography of Glycinamide Ribotides A and B, Related Compounds, and Reference Compounds

	R_F of compounds					
	Solvent 1	Solvent 2	Solvent 3	Solvent 4	Solvent 5	Solvent 6
Glycinamide ribotide A*	0.15	0.53	0.15	0.22	0.55†	
" " B*	0.15	0.53	0.15	0.22	0.55†	
" " hydrolyzed‡	0.38§	0.64§				0.31,§ 0.54‖
Glycinamide riboside	0.26¶			0.60¶**	0.77†	
				−0.82		
Glycine	0.38	0.60		0.48**	0.77	0.31
				−0.60		
Ribose-5-phosphate	0.26	0.37	0.07	0.28	0.60	0.12
Orthophosphate	0.56	0				

* Fractions isolated from Dowex 1 (acetate form) resin (see "Methods"). The radioactivity, periodate reaction, ninhydrin reaction (yellow), and organic phosphate all coincided.

† No periodate reaction because of the borate complex.

‡ 1 N HCl, 2 hours, 100°.

§ The radioactivity and ninhydrin reaction (blue) coincided.

‖ Pentose-reducing sugar only (apparently ribose).

¶ The radioactivity, ninhydrin color, and periodate reaction of the spot coincided.

** The R_F values were variable in this solvent.

salt was prepared by adjusting the solution to pH 9, adding an excess of $CaCl_2$, and precipitating with 4 volumes of ethanol.

Preparation of Glycinamide Ribotide Derivatives—Glycinamide riboside was prepared by the following procedure. 6.2 μmoles of glycinamide ribotide were incubated with 2.0 mg. of intestinal phosphatase and 3.0 ml. of 0.1 M Tris buffer at pH 8.0 for 80 minutes. A quantitative conversion to riboside occurred. After inactivation of the phosphatase by boiling, the

TABLE II

Analysis of Glycinamide Ribotide A

Component	Concentration	Molar ratio*
	μmoles per ml.	
Organic phosphate	1.35	1.00
Total nitrogen	2.84	2.12
Acid-labile nitrogen†	1.38	0.99
Pentose		
By orcinol	0.82	0.59
" periodate consumption	1.35	1.00
Reducing sugar		
Before hydrolysis	0	0
After " †	1.34	0.99
Glycine		
By Method 1‡	1.38	1.02
" " 2†	1.40	1.04

All determinations were made in duplicate on the glycinamide ribotide preparation described in the text.

* Calculated to a value of 1.00 for organic phosphate.

† Sample in N HCl for 2 hours at 100°. See Fig. 3 for the rate of ammonia liberation under these conditions.

‡ Glycinamide ribotide and glycine showed the same molar extinction with the Moore and Stein ninhydrin reagent (19).

solution was adjusted to pH 1.8 and passed through a Dowex 50 column (NH_4^+ form, 0.64 sq. cm. × 2.0 cm.) to adsorb the glycinamide riboside. The column was washed with water and with 0.01 N HCl, and then the glycinamide riboside was eluted with 0.03 M ammonium hydroxide. This fraction was lyophilized to dryness and taken up in water.

DNP-glycinamide ribotide was formed by reaction of the C^{14}-labeled glycinamide ribotide with 0.02 M 1-fluoro-2,4-dinitrobenzene in 0.05 M $KHCO_3$ and 25 per cent ethanol for 1 hour at 38° (21). The yellow DNP-glycinamide ribotide was separated from the excess reagent (after removal of ethanol) by extraction of the FDNB with ether and by paper chromatography with Solvent 7. In this solvent, DNP-glycinamide ribotide did

not migrate from the starting line. FDNB migrated to R_F 0.70 and authentic DNP-glycine to R_F 0.31.

Formylglycinamide ribotide was prepared by formylation of glycinamide ribotide at 45° by the procedure of Flynn *et al.* (22).

Results

Chromatographic Behavior of Glycinamide Ribotide and of Its Hydrolytic Products—Paper chromatographic studies, summarized in Table I, demonstrated that glycinamide ribotides A and B migrated as single compounds in five different solvents. The location of the radioactivity, non-reducing sugar, ninhydrin-reacting material, and organic phosphate all coincided. Glycine was the only amino acid liberated on acid hydrolysis as determined by paper chromatography (Table I).

Analysis of Glycinamide Ribotide—The calcium salt of glycinamide ribotide was analyzed for carbon, hydrogen, and ignited residue. The results of the analysis corresponded to the empirical formula:

$C_7H_{13}O_8N_2PCa \cdot \frac{1}{2}Ca(OH)_2$ (358).	Calculated.	C 23.3, H 3.88
	Found.[3]	" 23.12, " 3.79

Residue ($Ca_2P_2O_7 + CaO + H_2O$). Calculated, 44.3; found, 44.43

The molar ratios of different moieties of glycinamide ribotide compared to organic phosphate are shown in Table II. These data are for the glycinamide ribotide sample from which the calcium salt was prepared. These ratios may be summarized as follows: organic phosphate-total nitrogen-acid-labile nitrogen-pentose (by periodate method) 1:2:1:1. It is not clear why the orcinol method gave low values for the pentose. Prior hydrolysis of the compound for 40 minutes with 1.5 N HCl or heating with the orcinol reagent for 90 minutes did not increase the color. Ribose-5-phosphate gave the same color value as AMP-5.

On acid hydrolysis (1 N HCl, 2 hours, 100°) 1 mole of glycine was liberated per mole of organic phosphate. Glycine was identified by its migration on paper in three different solvent systems and by its degradation to formaldehyde by the ninhydrin reaction (20). Acid hydrolysis liberated 1 mole of reducing sugar and 1 mole of NH_3 per mole of organic phosphate. The rate of liberation of NH_3 is shown in Fig. 3. 2 hours were required for complete hydrolysis.[4]

The organic phosphate of glycinamide ribotide was slowly hydrolyzed by

[3] This analysis was made by the Huffman Microanalytical Laboratories, Wheatridge, Colorado.

[4] The slow rate of hydrolysis suggests that the glycosidic linkage in glycinamide ribotide is more stable than that of AMP-5 which, under the same conditions, is hydrolyzed quantitatively to adenine and ribose-5-phosphate in 30 minutes.

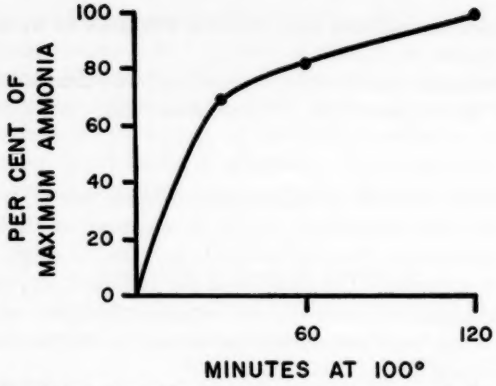


FIG. 3. Rate of formation of ammonia from glycinamide ribotide by hydrolysis with N HCl. 0.81 μmole of glycinamide ribotide (by phosphate analysis) was hydrolyzed in a volume of 0.7 ml. The maximal value for ammonia should be 0.81 μmole. (See Table II.)

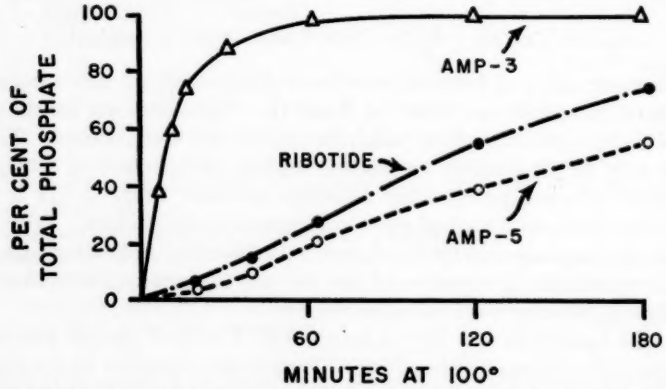


FIG. 4. Rate of hydrolysis of phosphate of glycinamide ribotide in N HCl. Glycinamide ribotide A was used (see the text). 0.25 μmole of each compound based on the total organic phosphate analysis was hydrolyzed in a final volume of 0.6 ml. of 1 N HCl.

heating with acid (Fig. 4). The rate of liberation of this phosphate is consistent only with a 5-phosphate ester (23, 24).

Derivatives of Glycinamide Ribotide—The dinitrophenyl derivative of C^{14}-glycinamide ribotide migrated as a single component in Solvents 7 and 8. The C^{14} activity and the color of the derivative were superimposed in each solvent. The R_F value of DNP-glycinamide ribotide in Solvent 8

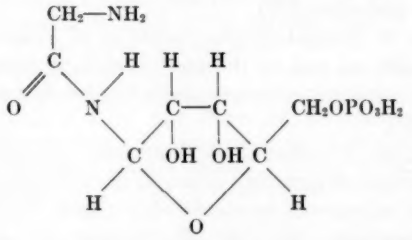
was 0.39, while that of DNP-glycine was 0.68. In 1.4 per cent $NaHCO_3$ solution the DNP-ribotide, purified by paper chromatography, exhibited a maximal molar extinction coefficient of 15,000 between 350 and 355 mμ, based on the organic phosphate analysis. DNP-glycine shows an E_{350} of 15,500 (25).

Glycinamide riboside migrated as a single component in each of three solvents (Table I). The position of the non-reducing sugar and the ninhydrin-reacting material corresponded exactly to the radioactivity. Reducing sugar and free glycine were absent, but by acid hydrolysis (1 N HCl, 100°, 2 hours), reducing sugar and glycine were liberated in the ratio 1.12:1.

Chemical formylation of glycinamide ribotide yielded a compound which behaved on an anion exchange column exactly as formylglycinamide ribotide prepared enzymatically. It was eluted from a Dowex 1 formate column by 0.05 M ammonium formate buffer, pH 5.0, whereas the unformylated compound was eluted at pH 6.5 (1–3).

DISCUSSION

This aliphatic ribotide precursor of inosinic acid has been assigned the following structure: *N*-glycyl-5-phosphoribofuranosylamine.



The purity of the isolated compound is based on (1) the good agreement of the elemental analysis of the calcium salt with the empirical formula, (2) the analysis of liberated glycine, organic phosphate, pentose, total nitrogen, and acid-labile nitrogen, (3) the chromatographic evidence, and (4) the constancy of C^{14} activity to pentose values in the fractions eluted from the Dowex 1 columns.

Four experimental facts have led to the conclusion that the amino group is unsubstituted. First, this compound reacted with ninhydrin to give a blue color without decarboxylation of the carbonyl carbon (19). Second, dinitrofluorobenzene reacted readily with the compound. Third, the mild conditions employed for formylation of the compound would be expected to result in substitution of the amino and not the amido nitrogen. Fourth, the chemical formylation of the compound yielded a derivative with different charge characteristics. This derivative behaved on anion exchange

columns as though it had one more net negative charge than glycinamide ribotide and corresponded exactly with enzymatically synthesized formylglycinamide ribotide (1–3). This could be accounted for only by formylation of the amino group. These experiments are in agreement with titration studies which show that formylglycinamide ribotide does not have a free primary amino group (26).

Evidence that the amide nitrogen is in glycosyl linkage with the pentose is based on several considerations. Acid hydrolysis of the ribotide liberated, in molar proportions, glycine, NH_3, and a reducing group. The rate of acid hydrolysis of the compound as measured by ammonia liberation (Fig. 3) was slow compared to the rate of hydrolysis of free glycinamide. The evidence given for the presence of a free amino group was not consistent with a glycosyl linkage to that nitrogen atom.

Ribose-5-phosphate is a component of the ribotide. Thus the only pentose compound present on a Dowex 1 borate column after hydrolysis of formylglycinamide ribotide was ribose-5-phosphate (1). The rate of acid hydrolysis of the phosphate of glycinamide ribotide indicated a 5- rather than a 2- or 3-phosphate ester (Fig. 4). The spectrum of the orcinol reaction with glycinamide ribotide was characteristic of an aldopentose and not of a ribulose derivative (27).

The similarity of chromatographic behavior of Fractions A and B of glycinamide ribotide as well as the analytical data suggested that these fractions were isomeric forms comparable to the two forms of formylglycinamide ribotide (28).

Recently Hartman, Levenberg, and Buchanan (26) have presented evidence for the structure of formylglycinamide ribotide, which, together with the unformylated compound, accumulated in pigeon liver preparations in the presence of azaserine.

SUMMARY

A procedure is described for the isolation and purification of glycinamide ribotide. Under these conditions it occurred in two isomeric forms.

Elemental analysis of the compound was in agreement with the structure, *N*-glycyl-5-phosphoribofuranosylamine. This compound contained molar proportions of glycyl, amido, pentose, and phosphate ester moieties. The pentose phosphate had the properties of ribose-5-phosphate. The glycosidic linkage of glycinamide ribotide was more stable than that of adenylic acid.

Glycinamide riboside was prepared from the ribotide by treatment with intestinal phosphatase. The dinitrophenyl derivative and the calcium salt of the ribotide have been prepared. Mild chemical formylation of glycinamide ribotide yielded a compound which was comparable in chroma-

tographic behavior to enzymatically synthesized formylglycinamide ribotide.

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XUM

THE EFFECT OF ADRENALECTOMY AND OF CORTISONE ADMINISTRATION ON THE METABOLISM OF L-TRYPTOPHAN*

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Paper chromatography has provided an excellent method for the detection and identification of tryptophan and its fluorescing metabolites (1) and has proved helpful in the development of methods for the quantitative colorimetric estimation of tryptophan, kynurenine, and anthranilic acid (2). The purpose of this communication is to report the use of these techniques in determining the effect of adrenalectomy and cortisone administration upon the metabolism of L-tryptophan in the rat, as judged from urinalysis after feeding L-tryptophan and from the metabolism of L-tryptophan and L-kynurenine by liver and kidney slices.

According to Porter, Stoerk, and Silber (3), adrenalectomy decreases the kynurenic acid output in the rat fed tryptophan, but does not alter the output of kynurenine and of xanthurenic acid. The administration of cortisone acetate to the adrenalectomized rat restores the kynurenic acid excretion to normal but does not affect the output of kynurenine and of xanthurenic acid. They therefore assumed that the effect of the hormone was to increase the rate of deamination or transamination whereby kynurenine was converted to kynurenic acid.

In tests involving the use of liver homogenates from normal and adrenalectomized rats, Knox observed that the production of kynurenine from DL-tryptophan was smaller with the latter animals (4). Subsequently, Knox and Auerbach reported that they were unable to find any other product of L-tryptophan oxidation besides kynurenine by methods which should have indicated the presence of anthranilic or kynurenic acid (5). Previous observations from our laboratory had indicated that both of these metabolites and kynurenine were formed by normal liver homogenates (2) and by normal liver slices (1) under conditions apparently similar to those used by Knox and Auerbach (5).

EXPERIMENTAL

In the present studies on whole animals, L-tryptophan was fed as the source of the excreted metabolites, and in the tissue slice tests both L-tryp-

* The data in this paper are taken from a dissertation submitted in August, 1955, in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa.

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tophan and L-kynurenine, the latter in the form of the sulfate, were employed as substrates. Kynurenic acid, xanthurenic acid, anthranilic acid, and hydroxyanthranilic acid were also used in the tissue tests, primarily as references in the paper chromatography and in the checks of the analytical procedures.

Materials

The L-tryptophan was prepared from *N*-acetyl-DL-tryptophan[1] by resolution with brucine and hydrolysis (6). Its $[\alpha]_D^{25}$ was $-30.7°$ ($c = 0.5$ per cent in water). The anhydrous L-kynurenine sulfate employed, which was prepared from acetyl-L-tryptophan by the method of Warnell and Berg (7), melted at 178° and showed $[\alpha]_D^{25}$ $+10.7°$. Values in literature for the latter range from $+8.5°$ to $+10.7°$ (7). The kynurenic acid, xanthurenic acid, and anthranilic acid were from lots previously described (1, 8). The 3-hydroxyanthranilic acid was synthesized (9) from 2-nitro-*m*-cresol by a method based in part on the procedure of Kenner and Turner (10).

The cortisone acetate[2] began to sinter at 228° and melted at 233–237°. A melting point of 235–238° has been reported (11). In a few tests, deoxycorticosterone acetate[2] was also employed, which melted at 154–156°. Melting points of 155–156° (12) and 157–159° (13) are recorded.

Preparation of Animals and Animal Tissues

Throughout the studies, rats of the Sprague-Dawley strain, 2 to 3 months of age and 180 to 260 gm. in weight, were housed in individual cages. The animals used in the urine studies were fed 10 gm. of a diet high in casein (14) per day throughout the experiment. The others were maintained on a stock diet (Arcady Rockland rat diet).

The adrenalectomies were performed under anesthesia by the lumbar approach under semisterile conditions. In the animals supplied the hormones, injections were begun on the day of the surgery. Two doses were given subcutaneously daily, 10 to 12 hours apart. Each dose contained 0.75 mg. of the hormone per 100 gm. of rat weight, as a 0.5 per cent suspension in a 1 per cent saline solution. The adrenalectomized rats were given a 1 per cent saline solution to drink, instead of the tap water allowed the control animals which had been subjected to a sham operation.

4 days after the adrenalectomy or the sham operation, 100 mg. of L-tryptophan were given by stomach tube per 100 gm. of body weight, in the form of a 2.5 per cent supersaturated aqueous solution. The animals were

[1] We are indebted to The Dow Chemical Company for this derivative.

[2] The cortisone and deoxycorticosterone acetates were made available to us through the generosity of Dr. Curt C. Porter of the Merck Institute for Therapeutic Research, Rahway, New Jersey.

transferred to metabolism cages treated with paraffin to permit the collection of urine. Prior to analysis, the urine and washings were concentrated by vacuum distillation, made up to 25 ml., and stored in the refrigerator under toluene.

In the studies involving liver and kidney slices, the animals received no tryptophan supplement. They were killed by a blow on the head 4 or 5 days after the operation. The livers and kidneys were removed immediately, chilled in chipped ice, and placed in cold Krebs-Ringer phosphate (0.1 M) solution, pH 7.4. Slices 0.3 to 1.0 mm. thick were prepared with the Stadie-Riggs slicer. Of these, 400 mg. (wet weight) were washed in cold buffer solution and incubated at 37° for 3 hours in Warburg vessels in an atmosphere of O_2 in a medium consisting of 2 ml. of Krebs-Ringer solution (pH 7.4) plus 1 ml. of buffered substrate, which contained 1 mg. of L-tryptophan or 0.645 to 1.0 mg. of L-kynurenine, as the sulfate. At the end of the incubation period, aliquots of 2 ml. were deproteinized with 2 ml. of 20 per cent trichloroacetic acid solution and made up to 10 ml. with water. The deproteinized solutions were then assayed for the remaining tryptophan and for kynurenine and kynurenic acid. The media used in the chromatographic tests were not previously freed of protein.

Analytical Procedures

The chromatographic tests (1) were carried out on 10 μl. of urine or 50 μl. of untreated medium. The fluorescing metabolites were identified by position and by color. Comparative chromatographic tests were made simultaneously with solutions of the pure reference compounds. Tryptophan was identified by the use of *p*-dimethylaminobenzaldehyde (15), with which it gave a purple spot.

In the analyses of the urine, attempts were first made to apply the methods of Mason and Berg for measuring kynurenine, anthranilic acid, and tryptophan (2). Good recoveries were obtained when these were added to the urine of animals fed a normal diet, but there is some uncertainty that kynurenine and anthranilic acid are the only diazotizable metabolites present in urines of animals fed L-tryptophan. No tryptophan could be detected chromatographically in such urines. When they were extracted at pH 4.0 with ether, which removes anthranilic acid but not kynurenine (16), and the aqueous residue was diazotized and incubated to destroy the kynurenine (2), only a trace of the original diazotizable fraction remained. The ether extract showed only the fluorescing spot characteristic of anthranilic acid. It is, nevertheless, possible that variable amounts of a diazotizable metabolite could have been excreted which did not fluoresce. Indole and indolepyruvic acid were excluded because they gave deep red-violet solutions immediately with the nitrous acid. Indolealdehyde and

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skatole gave faint responses only after the coupling agent was added, but it is unlikely that appreciable amounts of either could have been present in the urine. Unfortunately, no indican was available for testing. Kynurenic acid was estimated gravimetrically after precipitation from the acidified urine (17). Xanthurenic acid was not estimated; the paper chromatograms showed that enough was present to give a faint spot.

A second series of urine analyses was run in which kynurenine, kynurenic acid, and xanthurenic acid were determined by procedures essentially those employed by Porter, Stoerk, and Silber (3). In this series, kynurenine was estimated by precipitating it from the urine with mercuric sulfate and boiling the precipitate with alkali to liberate ammonia (18). With increasing amounts of added kynurenine sulfate, recoveries decreased from 90 to 65 per cent. Kynurenic acid was precipitated by acidifying the urine, oxidizing the precipitate to anthranilic acid with 30 per cent H_2O_2, and estimating the latter (19) by diazotization and coupling. With pure kynurenic acid, 97 to 99 per cent recoveries of the added compound were obtained. In the analysis for xanthurenic acid, (3), the urine was extracted with isoamyl alcohol and a green color formed with ferric chloride. Recoveries of 90 to 105 per cent of added xanthurenic acid were found possible.

In the tissue studies, tryptophan, kynurenine, and anthranilic acid were all found to respond to the diazotization and coupling techniques described by Mason and Berg (2). With the smaller amounts of substrate used in the current studies, too little anthranilic acid was produced for reliable estimation. Paper chromatograms showed that it was present in all the tests with liver tissue. Kynurenine was determined as originally described (2). In the estimation of tryptophan, kynurenine is first destroyed by diazotization and incubation at 37° for 1 hour to decompose the diazonium salt (2). Addition of 0.5 ml. of 0.4 per cent ammonium sulfamate immediately prior to the incubation was found to improve the subsequent estimation of tryptophan. To compensate for the extra volume, 0.5 ml. of 8 per cent sulfamate, instead of 1 ml. of 4 per cent, was added to destroy the larger excess of nitrite used in the diazotization of the tryptophan. When kynurenine was added in amounts likely to be encountered, 98 to 104 per cent could be recovered; with tryptophan, recoveries accounted for 94 to 100 per cent of the amount added. Kynurenic acid was estimated from the ultraviolet absorption at 312 mμ in the Beckman model DU spectrophotometer. Xanthurenic acid in trichloroacetic acid solution also absorbs at this wave-length, but, in agreement with previous observations (1), chromatographic evidence indicated that none was formed by liver tissue. Some is produced from kynurenine by kidney slices (1), but at 312 mμ the absorption by xanthurenic acid approaches its minimal value.

Since its absorption at 312 mμ is only a fourth as strong as that of kynurenic acid and the chromatographic evidence showed that only small amounts were present, the estimates of kynurenic acid production from kynurenine by kidney slices were at least approximately quantitative.

TABLE I

*Effect of Cortisone Acetate on Urinary Excretion of Tryptophan Metabolites by Adrenalectomized Rats and Rats Subjected to Sham Operation**

Treatment	Series	No. of rats	Metabolite excretion, mg. in 24 hrs.			
			Kynurenine	Kynurenic acid	Xanthurenic acid	Total diazotizable substance, calculated as anthranilic acid
Control	A	2	2.1 ± 0.7	15.0†		7.3 ± 3.5
	B	2	3.3 ± 0.4	24.0 ± 4.9	5.2 ± 0.2	
Control + cortisone acetate	A	3	3.4 ± 0.7	24.1 ± 6.4		13.4 ± 3.3
	B	3	2.9 ± 1.4	50.1 ± 13	4.9 ± 0.0	
Adrenalectomized	A	4	1.3 ± 0.2	2.2 ± 0.5		3.4 ± 0.8
	B	2	3.9 ± 1.0	11.4 ± 1.8	3.5 ± 1.4	
Adrenalectomized + cortisone acetate	A	5	2.8 ± 1.0	17.6 ± 9.4		11.4 ± 4.5
	B	2	5.0 ± 0.4	49.7 ± 6.6	5.3 ± 0.5	

* Each rat was given 100 mg. of L-tryptophan by stomach tube (2.5 per cent supersaturated solution in water) per 100 gm. of body weight in a single dose. The cortisone acetate was given in two daily doses of 0.75 mg. per 100 gm. of body weight in 0.5 per cent suspension in 1 per cent saline, for 4 days prior to the urine collection. In Series A, the analytical procedures of Mason and Berg (2) were employed; in the Series B, those of Porter, Stoerk, and Silber (3). ± = standard deviation of the mean.

† Only one analysis was made here.

Results

The data obtained in the two urinalysis series are summarized in Table I. They confirm, beyond a doubt, that adrenalectomy lowers the over-all capacity of the rat to produce anthranilic acid as the chief diazotizable substance and to yield kynurenic acid, and that the administration of cortisone, even to the control rat, increases the output of both of these. The data for excretion of kynurenine in the two series are not consistent, but, since both kynurenic acid and anthranilic acid are derived from kynurenine, a change in either implies a corresponding intermediate involvement of kynurenine. Xanthurenic acid also has its origin in kynurenine, but its output showed only minor and inconsistent changes.

In the tissue slice tests, L-kynurenine was even more readily attacked by liver from the adrenalectomized rat than by liver from the control rat, or

TABLE II

*Effect of Cortisone Acetate upon Degradation of L-Kynurenine by Liver Slices**

Four rats were employed in each group of tests.

Treatment	Kynurenine recovered, γ per 400 mg. tissue
Control	328 ± 19†
Adrenalectomized	238 ± 20
" + cortisone acetate‡	300 ± 35

* 645 γ of L-kynurenine (1000 γ of L-kynurenine sulfate·H_2O) were incubated in 3 ml. of Krebs-Ringer phosphate solution, pH 7.4, for 3 hours in O_2 at 37°, with 400 mg. (wet weight) of liver slices. Duplicate tests were always carried out.

† ± = standard deviation of the mean.

‡ 0.75 mg. of cortisone acetate (0.5 per cent suspension in 1 per cent saline) per 100 gm. of body weight was injected subcutaneously twice daily during the 4 days immediately following the adrenalectomy and immediately prior to the sacrifice of the rats.

TABLE III

*Effect of Cortisone Acetate (COA) and Deoxycorticosterone Acetate (DOCA) on Accumulation of Kynurenine and Kynurenic Acid in Tryptophan Media Incubated with Rat Liver Slices**

Treatment	No. of rats	Tryptophan recovered, γ per 400 mg. tissue	Kynurenine found, γ per 400 mg. tissue	Kynurenic acid found, γ per 400 mg. tissue
Control	11	385 ± 71†	134 ± 55†	74 ± 10†
	5	398 ± 34	143 ± 24	
Adrenalectomized	9	359 ± 105	72 ± 24	
	4			37 ± 3
Adrenalectomy + COA‡	5	442 ± 70	279 ± 51	
	4	400 ± 24	368 ± 26	145 ± 18
Adrenalectomy + DOCA‡	4	406 ± 50	77 ± 16	49 ± 18
Normal	15	399 ± 36	132 ± 21	
	4			53 ± 15
Normal + COA‡	9	345 ± 75	302 ± 39	
	4			108 ± 9
Normal + DOCA‡	7	421 ± 46	99 ± 11	53 ± 11
	4			

* 1000 γ of L-tryptophan were incubated in duplicate in 3 ml. of Krebs-Ringer phosphate solution, pH 7.4, for 3 hours in O_2 at 37°, with 400 mg. (wet weight) of liver slices.

† ± = standard deviation of the mean.

‡ 0.75 mg. of the hormone (0.5 per cent suspension in 1 per cent saline) per 100 gm. of body weight was injected subcutaneously twice daily for 4 days prior to sacrificing the rats. In the adrenalectomized rats, the injections were begun immediately following the operation.

from the rat provided cortisone twice daily after adrenalectomy (see Table II). The amount of L-tryptophan metabolized by liver tissue in 3 hours was in all instances about the same (Table III). Contrary to the findings of Knox and Auerbach (5), not only kynurenine but also kynurenic acid and other metabolites were produced. Anthranilic acid was found consistently on paper chromatograms, but, with the small amounts of L-tryptophan employed as substrate, too little was produced for accurate

TABLE IV

Effect of Cortisone Acetate (COA) and Deoxycorticosterone Acetate (DOCA) on Utilization of L-*Kynurenine by Rat Kidney Slices and Accumulation of Kynurenic Acid*

Series No.*	Treatment	No. of rats	Kynurenine recovered, γ per 400 mg. tissue	Kynurenic acid found, γ per 400 mg. tissue
I	Normal	11	302 ± 32†	
		4		345 ± 28
	Normal + COA‡	10	310 ± 36	
		4		338 ± 30
	Normal + DOCA‡	7	288 ± 32	
		4		349 ± 15
II	Control	3	148 ± 12	
		2	150 ± 0	232 ± 15
	Adrenalectomized	3	168 ± 23	
		4	123 ± 24	246 ± 9
	Adrenalectomized + COA‡	3	131 ± 20	
		4	138 ± 14	222 ± 17

* In Series I, 1000 γ of L-kynurenine were incubated in duplicate in 3 ml. of Krebs-Ringer phosphate buffer solution, pH 7.4, for 3 hours in O_2 at 37°, with 400 mg. (wet weight) of kidney slices. In Series II, conditions were the same, but only 645 γ of L-kynurenine were employed per test.

† ± = standard deviation of the mean.

‡ The hormones were administered as indicated in Table III.

quantitative estimation. Less kynurenine (4, 5) and less kynurenic acid accumulated when L-tryptophan was incubated with liver tissue from the adrenalectomized rat than with liver tissue from the control or normal rat. Cortisone acetate, but not deoxycorticosterone acetate, stimulated the production of these metabolites. Addition of cortisone to the media and its injection into the normal rat 1 hour before sacrifice were both tried and found to be without appreciable effect on the capacity of the liver to degrade tryptophan or convert it to kynurenine or kynurenic acid.

The data outlined above fail to support the assumption that cortisone increases the oxidation of tryptophan by the liver slice to kynurenine *per se* (4), or that it favors primarily the transamination of kynurenine to

kynurenic acid. They seem rather to warrant the assumption that cortisone (*a*) favors the degradation of tryptophan via kynurenine, possibly by inhibiting other oxidative routes, (*b*) impedes the degradation of kynurenine by routes which do not yield kynurenic acid, or (*c*) does both.

Kidney slices were found to produce no fluorescing metabolites from L-tryptophan, of which 67 to 71 per cent could be recovered after incubation under conditions the same as those used with liver slices. Kidney tissue is known to produce indolepyruvic acid from tryptophan (1) as a non-fluorescing metabolite. When kidney slices were incubated with L-kynurenine, greater degradation occurred than with liver slices, and large amounts of kynurenic acid were formed (Table IV). A small amount of xanthurenic acid and traces of several unidentified fluorescing metabolites were also detected by paper chromatography. No anthranilic acid was found. No significant qualitative or quantitative alterations of metabolism could be detected, as judged by kynurenic acid assay or from number or area and intensity of the fluorescing spots on the paper chromatograms.

SUMMARY

Adrenalectomy decreases the output of kynurenic acid and of total diazotizable substances (including kynurenine) in the rat fed L-tryptophan. Both are increased by the injection of cortisone acetate in the adrenalectomized animal and in the animal subjected to a sham operation.

Comparative tests with liver slices from adrenalectomized rats, from such rats injected with cortisone acetate, and from control rats showed that adrenalectomy did not alter the quantity of L-tryptophan degraded, but did decrease the accumulation of kynurenine and kynurenic acid. It increased the degradation of L-kynurenine. Cortisone is tentatively assumed either to inhibit the degradation of L-tryptophan by pathways which do not yield kynurenine, to inhibit the degradation of L-kynurenine by pathways which do not produce kynurenic acid, or to do both.

In analogous tests with kidney slices from comparably prepared rats, essentially no differences in metabolism were found. Neither the metabolism of L-tryptophan by liver tissue nor that of L-kynurenine by kidney tissue was appreciably affected by the administration of deoxycorticosterone acetate.

No appreciable alteration by adrenalectomy could be detected in the qualitative pattern of fluorescing metabolites by the routine application of paper chromatography.

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